



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/85, 15/86, 15/62, 15/12, 5/10,</b> <b>C07K 14/60, A01K 67/027, A61K 47/32,</b> <b>48/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 99/05300</b> <b>(43) International Publication Date:</b> 4 February 1999 (04.02.99)
<b>(21) International Application Number:</b> PCT/US98/15434 <b>(22) International Filing Date:</b> 24 July 1998 (24.07.98)  <b>(30) Priority Data:</b> 60/053,609                      24 July 1997 (24.07.97)                      US 60/062,608                      20 October 1997 (20.10.97)                      US  <b>(71) Applicant (for all designated States except US):</b> GEN- EMEDICINE, INC. [US/US]; 8301 New Trails Drive, The Woodlands, TX 77381-4248 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SCHWARTZ, Robert, J. [US/US]; 4019 Marlowe, Houston, TX 77005 (US). DRAGHIA-AKLI, Ruxandra [RO/US]; 2255 Braeswood Park Drive #159, Houston, TX 77030 (US). LI, Xuyang [CN/US]; 2111 Holly Hail #310, Houston, TX 77054 (US). EASTMAN, Eric, M. [US/US]; 7465 Mink Hollow Road, Highland, MD 20777 (US).  <b>(74) Agents:</b> WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished          upon receipt of that report.</i>
<b>(54) Title:</b> GHRH EXPRESSION SYSTEM AND METHODS OF USE  <b>(57) Abstract</b> <p>This invention relates to gene delivery and expression, including gene therapy, by using vectors which establish controlled expression of recombinant GHRH genes within tissues at certain levels. The expression vectors of the present invention can also be regulated by a regulatory system and/or constructed with a coating. The invention concerns the use of plasmid cDNA containing nucleotide sequences encoding species-specific GHRH polypeptides and teaches expression of these polypeptides in a variety of animal species and animal tissues to provide stimulation of GH at physiological levels, sufficient to enhance growth and improve the immune systems of agricultural and other domesticated species, including household pets. The invention also concerns a novel method for reducing the rate of proteolytic degradation of secreted GHRH.</p>		

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**DESCRIPTION****GHRH EXPRESSION SYSTEM AND METHODS OF USE**

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The work herein was supported by grants from the United States Government. The United States Government may have certain rights in the invention.

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**Statement of Related Applications**

This application claims priority to U.S. Patent Application No. 60/062,608, filed October 20, 1997 and U.S. Patent Application No. 60/053,609, filed July 24, 1997, both of which are incorporated herein by reference  
15 in their entirety, including any drawings.

**Field of the Invention**

The present invention is in the field of recombinant DNA technology. This invention relates to vectors which encode stable messenger RNA (mRNA) and  
20 methods of using such vectors. In particular, this invention relates to vectors which establish controlled expression of recombinant genes within a tissue; the expression may be at levels which are useful for gene therapy and other applications. The invention further  
25 relates to vectors able to express growth hormone releasing hormone (GHRH) and gene sequences inserted into vectors that control the production of growth hormone releasing hormone in non-human vertebrate animals. The invention is directed further to the use  
30 of these vectors in the respective non-human animals to further growth and strengthen their immune systems.

### Background of the Invention

None of the information provided herein is admitted to be prior art to the present invention, but is  
5 provided only to aid the understanding of the reader.

Growth hormone (GH) secretion by the anterior pituitary is stimulated by growth hormone releasing hormone (GHRH) and inhibited by somatostatin (SS), both hypothalamic hormones (Scanlong, M.F. et al., 1996,  
10 *Hormone Research* 149-154). GH enhances protein synthesis, lypolysis, and epiphyseal growth, and is implicated in the regulation of the immune system. GH increases circulating insulin-like growth factor I (IGF-1) levels, which in turn, mediates growth in the liver  
15 and peripheral tissues.

The GHRH-GH-IGF-I axis undergoes dramatic changes during the aging process and in the elderly (Iranmanesh et al., 1991, *J. Clin. Endocrin. & Metab.* 73:1081-1088; D'Costa A.P. et al., 1993, *J. Reproduction & Fertility -*  
20 *Suppl.* 46:87-98,) with decreased GH production rate and GH half-life, decreased IGF-I response to GH and GHRH stimuli that lead to osteoporosis, increase in fat and decrease in lean body mass and tissue function (Corpas et al., 1993, *Endocrine Rev.* 14:20-39).

25 In addition, genetic disorders of growth have also been ascribed to defects in the GHRH-GH-IGF-I axis, as those of GHRH receptor (Cao, Wagner, Hindmarsh, Eble, & Mullis, 1995, *Pediatr. Res.* 38:962-966), GH gene (Cogan et al., 1993, *J. Clin. Endocrin. & Metab.* 76:1224-1228;  
30 Vnencak-Jones et al., 1988, *PNAS* 85:5615-5619), GH receptor (Amselem et al., 1993, *Human Molec. Gen.* 2:355-359; Amselem et al., 1991, *Paediatrica Scandinavica* -

Supplement 377:81-86; Meacham et al., 1993, *J. Clin. Endocrin. & Metab.* 77:1379-1383) and pit-1 (Parks et al, 1993, *Hormone Research* 40:54-61) a pituitary specific transcription factor. In many cases growth retardation (GR) is a secondary manifestation of an unrelated primary affection (Turner syndrome, chronic renal failure, ovary resistant syndrome) or the exact cause of GR can not be established (Parks et al., in *Molecular Endocrinology: Basic Concepts and Clinical Correlations* (ed. Weintraub, B.D., Raven Press Ltd., New York, 1995) p.473-490).

In these cases of GR where the GHRH-GH-IGF-I axis is unaffected and in elderly, as well as in nonstatural related catabolic conditions (burn, sepsis, trauma associated pathology, chronic obstructive pulmonary disease), GH or GHRH replacement therapy is efficient.

Recombinant GH therapy is currently used in clinics, but a large number of studies have shown that side effects occur frequently, including edema, hypertension, carpal tunnel syndrome, hyperinsulinemia and impaired glucose tolerance (Marcus et al., 1990, *J. Clin. Endocrin. & Metab.* 70:519-527; Salomon et al., 1989, *New Engl. J. Med.* 321:1797-1803).

GH and IGF-1 also have beneficial effects on immune function (LeRoith, D. et al., *Endocrinology* 137:1071-1079 (1996)); Kotzmann, H. et al., *Neuroendocrinology* 60:618-625 (1994)). In farm animals, GHRH is galactopoietic (stimulates milk production) with no alteration in milk composition, increases the feed to milk conversion and sustains growth, mostly through increased lean body mass (Enright, W.J. et al., *Journal of Animal Science* 71:2395-2405 (1993); Enright, W.J. et

al., *Journal of Dairy Science* 69:344-351 (1986)).

Studies have shown that relatively small amounts of GHRH are required to stimulate the production and secretion of GH in all species. Some benefits of increasing GH in non-human vertebrate animals are improved growth rates, an increase in lean body mass, an increase in feed efficiency in pigs, beef cattle and sheep, increased milk production in dairy cows and goats, and enhanced production of lean meat and egg production in poultry.

GH also enhances the immune system in animals. In animals GHRH will have a great therapeutic utility in the treatment of cachexia in chronic diseases such as cancer, diabetes, due to growth hormone production abnormalities, enhancement of burn and wound healing, bone healing, retardation of the aging process and osteoporosis. However, the greatest use will be in agricultural animals. Intramuscular injection of DNA vector can persist for several months to produce sustained levels of GHRH. The intramuscular delivery of GHRH vector represents a practical method for improving performance in livestock animals.

Current limitations of recombinant GHRH therapy are the high cost of recombinant proteins, the short half-life of the peptides *in vivo* and the requirement for frequent administration (1-3 times/day) of either subcutaneous or intravenous injections. Using a GHRH injectable DNA plasmid based vector will enhance endogenous GH secretion in vertebrate animals with GH deficiencies in a manner more closely mimicking the natural process and in a less expensive manner than classical therapies.

### Summary of the Invention

The present invention is based in part on the identification of certain nucleic acid sequences which confer advantageous tissue targeting, expression, and secretion properties. Such sequences are utilized in the construction of plasmid vectors encoding GHRH, for delivery and expression of the GHRH coding sequences.

Expression of these vectors can be tissue specific.

These vectors are useful in facilitating enhanced expression in tissues as well as in targeting expression with tissue specificity. These vectors can be used to treat diseases by gene therapy by restricting expression of a gene encoded on the vector to targeted tissues. Vectors containing such sequences are able to provide gene delivery and controlled expression of recombinant genes within tissues; such expression can be at levels that are useful for gene therapy and other applications.

These vectors can also be used to create transgenic animals for research or livestock improvement.

The ability of the expression vector to provide enhanced product secretion as well as direct expression to specific tissues allows the vector to be used for treating numerous diseases. The above vectors can be used in gene therapy where a vector encoding a therapeutic product is introduced into a tissue so that the tissue will express the therapeutic product. For example, the above vectors may be used for treating muscle atrophy associated with neurological, muscular, or systemic disease or aging by causing tissues to express certain trophic factors.

In addition, the vectors can be used for gene replacement of inherited genetic defects or acquired hormone deficiencies, for vaccination in humans or

animals to induce immune responses, or for creating transgenic animals. The transgenic animals can be used as models for studying human diseases, for assessing and exploring novel therapeutic methods, to assess the effects of chemical and physical carcinogens, and to study the effect of various genes and genetic regulatory elements. Furthermore, transgenic animals can be used to develop commercially important livestock species. The above vectors can also be used to transform cells to produce particular proteins and RNA *in vitro*.

Expression of such vectors having a GHRH encoding sequence in the body of a vertebrate, e.g., a human, can produce both direct and indirect effects. The GHRH produces direct effects by the direct action of the GHRH polypeptide. However, indirect effects may also be produced due to the effect of the GHRH inducing or turning on the expression of other genes, or modulating the activity of other gene products. In particular, expression of GHRH can affect the levels of GH and IGF-I.

In a first aspect, the present invention features a vector for expression of a nucleic acid sequence in tissue by encoding stable mRNA. The vector includes a 5' flanking region which includes necessary sequences for the expression of a nucleic acid cassette, which include a promoter sequence, preferably an actin gene promoter sequence, more preferably a skeletal actin gene. The vector also includes a 3' flanking region, which includes a 3'UTR and/or a 3' NCR, which enhances secretion of the product expressed from the nucleic acid cassette. Preferably the 3' UTR is from the 3' region of a growth hormone gene, more preferably from a human growth hormone gene. Alternatively, in related vectors,



the 3' sequences may be selected to provide a higher level of retention of the product within a tissue, e.g., within a muscle tissue, rather than enhancing secretion. Such sequences can, for example, be from a skeletal  $\alpha$ -  
5 actin gene. The vector also includes a linker which connects the 5' flanking region to a nucleic acid. The linker does not contain the coding sequence of a gene that the linker is naturally associated with. That is, the linker is not the normal gene associated with the 5'  
10 and 3' regions. Preferably, the linker includes a sequence coding for a GHRH gene, more preferably human GHRH. The 3' flanking region is 3' to the position for inserting coding sequence or the nucleic acid cassette.

The term "flanking region" as used herein refers to  
15 nucleotide sequences on either side of an associated gene. Flanking regions can be either 3' or 5' to a particular gene in question. In general, flanking sequences contain elements necessary for regulation of expression of a particular gene. Such elements include,  
20 but are not limited to, sequences necessary for efficient expression, as well as tissue specific expression. Examples of sequences necessary for efficient expression can include specific regulatory sequences or elements adjacent to or within the protein  
25 coding regions of DNA. These elements, located adjacent to the gene, are termed *cis*-acting elements. The signals are recognized by other diffusible biomolecules in *trans* to alter the transcriptional activity. These biomolecules are termed *trans*-acting factors. *Trans*-  
30 acting factors and *cis*-acting elements have been shown to contribute to the timing and developmental expression pattern of a gene. *Cis*-acting elements are usually thought of as those that regulate transcription and are

usually found within promoter regions and within upstream (5') or downstream (3') DNA flanking regions.

Flanking DNA with regulatory elements that regulate expression of genes in tissue may also include  
5 modulatory or regulatory sequences which are regulated by specific factors, such as glucocorticoids, androgens, progestins, antiprogestins (PCT US93/04399; PCT US96/04324), vitamin D<sub>3</sub> and its metabolites, vitamin A and its metabolites, retinoic acid, calcium as well as  
10 others.

"Modulatory" or "regulatory" sequences as used herein refer to sequences which may be in the 3' or 5' flanking region, where such sequences can enhance activation and/or suppression of the transcription of  
15 the associated gene.

"Responsive" or "respond" as used herein refers to the enhancement of activation and/or suppression of gene transcription as discussed below.

"Metabolite" as used herein refers to any product  
20 from the metabolism of the regulatory factors which regulate gene expression.

In addition to the above, either or both of the 3' or 5' flanking regions can cause tissue specificity. Such tissue specificity provides expression  
25 predominantly in a specified cell or tissue.

"Predominantly" as used herein means that the gene associated with the 3' or 5' flanking region is expressed to a higher degree only in the specific tissue as compared to low expression or lack of expression in  
30 nonspecific tissue. The 3' or 5' flanking regions singularly or together as used herein may provide expression of the associated gene in other tissues but to a lower degree than expression in tissues or cells

where expression is predominate. Expression is preferentially in the specified tissue. Such predominant expression can be compared with the same magnitude of difference as will be found in the natural expression of the gene (i.e. as found in a cell *in vivo*) in that particular tissue or cell type as compared with other nonspecific tissues or cells. Such differences can be observed by analysis of mRNA levels or expression of natural gene products, recombinant gene products, or reporter genes. Furthermore, northern analysis, X gal immunofluorescence or CAT assays as discussed herein and as known in the art can be used to detect such differences.

The 3' flanking region contains sequences or regions, e.g. 3'UTR and/or 3' NCR, which regulate expression of a nucleic acid sequence with which it is associated. The 3' flanking regions can provide tissue-specific expression to an associated gene. The 3' flanking region also contains a transcriptional termination signal.

The term "3' flanking region" as used herein includes that portion of a naturally occurring sequence 3' to the transcribed portion of the gene which are responsible for mRNA processing and/or tissue-specific expression. That portion can be readily defined by known procedures. For example, the portions of a 3' flanking region which are responsible for mRNA stability and/or tissue-specific expression can be mapped by mutational analysis or various clones created to define the desired 3' flanking region activity in a selected vector system.

The 3' flanking region can contain a 3'UTR and/or a 3' NCR. The term "3' untranslated region" or "3'UTR"

refers to the sequence at the 3' end of structural gene which is transcribed from the DNA but not translated into protein. This 3'UTR region does not contain a poly(A) sequence, but generally contains a site at which  
5 a poly(A) sequence is added. Poly (A) sequences are only added after the transcriptional process.

Myogenic-specific 3'UTR sequences can be used to allow for specific stability in muscle cells or other tissues. As described below, myogenic-specific  
10 sequences refers to gene sequences normally expressed in muscle cells, e.g., skeletal, heart and smooth muscle cells. Myogenic specific mRNA stability provides an increase in mRNA stability within myogenic cells. The increase in stability provides increased expression.  
15 The 3'UTR and 3' NCR sequences singularly or together can provide a higher level of mRNA accumulation through increased mRNA stability. Thus, increased expression and/or stability of mRNA leads to increased levels of protein production.

20 The term "3' non-coding region" or "3'NCR" is a region which is adjacent to the 3'UTR region of a structural gene. The 3'NCR region generally contains a transcription termination signal. Once transcription occurs and prior to translation, the RNA sequence  
25 encoded by the 3'NCR is usually removed so that the poly(A) sequence can be added to the mRNA. The 3'NCR sequences can also be used to allow mRNA stability as described above. The 3'NCR may also increase the transcription rate of the nucleic acid cassette.

30 Either or both of the 3'UTR and 3' NCR sequences can be selected from a group of myogenic-specific genes. Examples of myogenic-specific genes include the skeletal  $\alpha$ -actin gene, fast myosin-light chain 1/3 gene,

myosin-heavy chain gene, troponin T gene, acetylcholine receptor subunit genes and muscle creatinine kinase gene.

In reference to 3' flanking regions of this invention, the term "growth hormone" refers to a gene product identified as a growth hormone, for example, human growth hormone or bovine growth hormone. Homologous gene sequences are known in the art for a variety of different vertebrate animals. In different embodiments, the vectors can incorporate 3' sequences, including 3' UTR sequences from such growth hormone genes. The 3' sequence can be modified from the sequence naturally found in the animal, for example by the deletion of ALU repeat sequence from human growth hormone 3' UTR. The deletion of ALU repeats or ALU repeat-like sequences can be performed with a variety of 3' sequences; such deletion generally reduces the rate of homologous recombination. A variety of other modifications may also be made without destroying the tissue targeting, stabilizing, and secretion properties of the 3' sequence.

The 5' flanking region is located 5' to the associated gene or nucleic acid sequence to be expressed. As with the 3' flanking region, the 5' flanking region can be defined by known procedures. For example, the active portion of the 5' flanking region can be mapped by mutational analysis or various clones of the 5' flanking region created to define the desired activity in a selected vector. The 5' flanking region can include, in addition to the above regulatory sequences or elements, a promoter, a TATA box, and a CAP site, which are in an appropriate relationship sequentially and positionally for the expression of an

associated gene.

In this invention, "sequences necessary for expression" are those elements of the 5' flanking region which are sequentially and positionally in an appropriate relationship to cause controlled expression of a gene within a nucleic acid cassette. Expression is controlled to certain levels within tissues such that the expressed gene is useful for gene therapy and other applications involving gene delivery. The 5' sequence can contain elements which regulate tissue-specific expression or can include portions of a naturally occurring 5' element responsible for tissue-specific expression.

The term "promoter," as used herein refers to a recognition site on a strand of DNA to which RNA polymerase binds. The promoter usually is a DNA fragment of about 100 to about 200 base pairs (in eukaryotic genes) in the 5' flanking DNA upstream of the CAP site or the transcriptional initiation start site. The promoter forms an "initiation complex" with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibitory sequences termed "silencers". The promoter can be one which is naturally (i.e., associated as if it were within a cell *in vivo*) or non-naturally associated with a 5' flanking region.

A variety of promoters can be used. Some examples include thymidine kinase promoter, myogenic-specific promoters including skeletal  $\alpha$ -actin gene promoter, fast myosin light chain 1 promoter, myosin heavy chain promoter, troponin T promoter, and muscle creatinine kinase promoter, as well as non-specific promoters

including the cytomegalovirus immediate early promoter, and Rous Sarcoma virus LTR. These promoters or other promoters used with the present invention can be mutated in order to increase expression of the associated gene.

- 5 Furthermore a promoter may be used by itself or in combination with elements from other promoters, as well as various enhancers, transcript stabilizers, or other sequences capable of enhancing function of the vector.

"Mutation" as used herein refers to a change in the  
10 sequence of genetic material from normal, causing a change in the functional characteristics of the gene. This includes gene mutations where only a single base is changed in the natural gene promoter sequences or multiple bases are changed.

- 15 The term "intron" as used herein refers to a section of DNA occurring in a transcribed portion of a gene which is included in a precursor RNA but is then excised during processing of the transcribed RNA before translation occurs. Intron sequences are therefore not  
20 found in mRNA nor translated into protein. The term "exon" as used herein refers to a portion of a gene that is included in a transcript of a gene and survives processing of the RNA in the cell to become part of a mature mRNA. Exons generally encode three distinct  
25 functional regions of the RNA transcript. The first, located at the 5' end which is not translated into protein, termed the 5' untranslated region (5' UTR), signals the beginning of RNA transcription and contains sequences that direct the mRNA to the ribosomes and  
30 cause the mRNA to be bound by ribosomes so that protein synthesis can occur. The second contains the information that can be translated into the amino acid sequence of the protein or function as a bioactive RNA

molecule. The third, located at the 3' end is not translated into protein, i.e. 3' UTR, contains the signals for termination of translation and for the addition of a polyadenylation tail (poly(A). In particular, the 3' UTR as defined above can provide mRNA stability. The intron/exon boundary will be that portion in a particular gene where an intron section connects to an exon section. The terms "TATA box" and "CAP site" are used as they are recognized in the art.

10       The term "linker" as used herein refers to DNA which contains the recognition site for a specific restriction endonuclease. Linkers may be ligated to the ends of DNA fragments prepared by cleavage with some other enzyme. In particular, the linker provides a recognition site for inserting the nucleic acid cassette which contains a specific nucleic sequence to be expressed. This recognition site may be but is not limited to an endonuclease site in the linker, such as Cla-I, Not-I, XmaI, Bgl-II, Pac-I, XhoI, NheI, Sfi-I. A linker can be designed so that the unique restriction endonuclease site contains a start codon (e.g. AUG) or stop codon (e.g. TAA, TGA, TCA) and these critical codons are reconstituted when a sequence encoding a protein is ligated into the linker. Such linkers commonly include an NcoI or SphI site.

25       The term "leader" as used herein refers to a DNA sequence at the 5' end of a structural gene which is transcribed and translated along with the gene. The leader usually results in the protein having an n-terminal peptide extension sometimes called a pro-sequence. For proteins destined for either secretion to the extracellular medium or the membrane, this signal sequence directs the protein into endoplasmic reticulum



from which it is discharged to the appropriate destination. The leader sequence normally is encoded by the desired nucleic acid, synthetically derived or isolated from a different gene sequence. A variety of leader sequences from different proteins can be used in the vectors of the present invention. Some non-limiting examples include gelsolin, albumin, fibrinogen and other secreted serum proteins.

The term "vector" as used herein refers to a nucleic acid, e.g., DNA derived from a plasmid, cosmid, phasmid or bacteriophage or synthesized by chemical or enzymatic means, into which one or more fragments of nucleic acid may be inserted or cloned which encode for particular genes. The vector can contain one or more unique restriction sites for this purpose, and may be capable of autonomous replication in a defined host or organism such that the cloned sequence is reproduced. The vector may have a linear, circular, or supercoiled configuration and may be complexed with other vectors or other materials for certain purposes. The components of a vector can include but are not limited to a DNA molecule incorporating: (1) a sequence encoding a therapeutic or desired product; and (2) regulatory elements for transcription, translation, RNA stability and replication.

The vector can be used to provide expression of a nucleic acid sequence in tissue. In the present invention this expression is enhanced by providing stability to an mRNA transcript from the nucleic acid sequence and/or secretion of the therapeutic protein. Expression includes the efficient transcription of an inserted gene or nucleic acid sequence within the vector. Expression products may be proteins including

but not limited to pure protein (polypeptide), glycoprotein, lipoprotein, phosphoprotein, or nucleoprotein. Expression products may also be RNA. The nucleic acid sequence is contained in a nucleic acid cassette. Expression of the nucleic acid can be continuous or controlled by endogenous or exogenous stimuli.

The term "control" or "controlled" as used herein relates to the expression of gene products (protein or RNA) at sufficiently high levels such that a therapeutic effect is obtained. Levels that are sufficient for therapeutic effect are lower than the toxic levels. Levels of expression for therapeutic effect within selected tissues corresponds to reproducible kinetics of uptake, elimination from cell, post-translational processing, and levels of gene expression, and, in certain instances, regulated expression in response to certain endogenous or exogenous stimuli (e.g., hormones, drugs).

The term "nucleic acid cassette" as used herein refers to the genetic material of interest which codes for a protein or RNA. The nucleic acid cassette is positionally and sequentially oriented within the vector such that the nucleic acid in the cassette can be transcribed into RNA, and when necessary, translated into a protein in the transformed tissue or cell. Preferably, the cassette has 3' and 5' ends adapted for ready insertion into a vector, e.g., it has restriction endonuclease sites at each end. In the vectors of this invention, a nucleic acid cassette contains a sequence coding for growth hormone releasing hormone (GHRH), e.g., human GHRH.

The term "tissue" as used herein refers to a

collection of cells specialized to perform a particular function or can include a single cell. The cells may be of the same type or of different types.

The term "gene", e.g., "myogenic genes," as used  
5 herein refers to those genes exemplified herein and their equivalence in other animal species or other tissues. Homologous sequences (i.e. sequences having a common evolutionary origin representing members of the same superfamily) or analogous sequences (i.e. sequences  
10 having common properties though a distinct evolutionary origin) are also included so long as they provide equivalent properties to those described herein. It is important in this invention that the chosen sequence provide the enhanced levels of expression, expression of  
15 the appropriate product, and/or tissue-specific expression as noted herein. Those in the art will recognize that the minimum sequences required for such functions are encompassed by the above definition. These minimum sequences are readily determined by  
20 standard techniques exemplified herein.

The term "myogenic" refers to muscle tissue or cells. The muscle tissue or cells can be *in vivo*, *in vitro*, or *in vitro* tissue culture and capable of differentiating into muscle tissue. Myogenic cells  
25 include skeletal, heart and smooth muscle cells. Genes are termed "myogenic" or "myogenic-specific" if they are expressed or expressed preferentially in myogenic cells. Vectors are termed "myogenic" or "myogenic-specific" if they function preferentially in myogenic muscle tissue  
30 or cells. Myogenic activity of vectors can be determined by transfection of these vectors into myogenic cells in culture, injected into intact muscle tissue, or injected into mammalian oocytes to be stably incorporated into

the genome to generate transgenic animals which express the protein or RNA of interest in myogenic cells.

The term "non-myogenic" refers to tissue or cells other than muscle. The tissues or cells can be *in vivo*,  
5 *in vitro*, or *in vitro* tissue culture.

In a preferred embodiment, the vector described above may have its 5' flanking region from myogenic genes, in particular the skeletal  $\alpha$ -actin gene, e.g., a chicken skeletal  $\alpha$ -actin gene. Specifically, this can  
10 include a promoter sequence which may be linked with other 5' UTR sequences, which can include an intron. While vectors using the chicken skeletal  $\alpha$ -actin promoter and/or other 5' flanking sequences are exemplified herein, the 5' sequences for  $\alpha$ -actin genes  
15 are highly conserved, therefore, such 5'  $\alpha$ -actin sequences can be utilized from other vertebrate species, including, for example, human.

In preferred embodiments, the 3' UTR is from a growth hormone gene, preferably from a human growth  
20 hormone gene, and preferably includes a poly(A) signal.

This sequence can be linked immediately following the natural translation termination codon for a cDNA sequence coding for the protein or RNA to be expressed.

As discussed above, these regions can be further and  
25 more precisely defined by routine methodology, e.g., deletion or mutation analysis or their equivalents.

The 5' or 3' sequences may have a sequence identical to the sequence as naturally found, but may also have changed sequences which provide equivalent  
30 function to a vector in which such 5' or 3' sequences are incorporated. Such a change, for example, could be a change of ten nucleotides in any of the above regions.

In particular, such changes can include the deletion of ALU repeat sequences from the 3' UTR. This is only an example and is non-limiting.

Also in preferred embodiments, the sequence  
5 encoding GHRH is a synthetic GHRH coding sequence. Such a synthetic sequence has a nucleotide sequence which differs from a natural human GHRH coding sequence. It is preferred that the sequence utilize optimal codon usage; preferably at least 50%, 70%, or 90% of the  
10 codons are optimized. Thus, in preferred embodiments the synthetic DNA sequence has at least 80, 90, 95, or 99% sequence identity to the sequence of **SEQ ID NO. 1**. In a particular preferred embodiment, the synthetic DNA sequence has at least 95% identity, more preferably at  
15 least 99% identity, and most preferably 100% identity to the sequence of **SEQ ID NO. 2**.

In addition, another embodiment of the above vector may contain a regulatory system for regulating expression of the nucleic acid cassette. The term  
20 "regulatory system" as used herein refers to *cis*-acting or *trans*-acting sequences incorporated into the above vectors which regulate in some characteristic the expression of the nucleic acid of interest as well as *trans*-acting gene products which are co-expressed in the  
25 cell with the above described vector. Regulatory systems can be used for up-regulation or down regulation of expression from the normal levels of expression or existing levels of expression at the time of regulation.

The system contributes to the timing and developmental  
30 expression pattern of the nucleic acid.

One construction of a regulatory system includes a chimeric *trans*-acting regulatory factor incorporating elements of a serum response factor capable of

regulating expression of the vector in a cell. The chimeric transacting regulatory factor is constructed by replacing the normal DNA binding domain sequence of the serum response factor with a DNA binding domain sequence of a receptor. The serum response factor has a *trans*-activation domain which is unchanged. The *trans*-activation domain is capable of activating transcription when an agent or ligand specific to the receptor binding site binds to the receptor. Thus, regulation can be controlled by controlling the amount of the agent.

The DNA binding domain sequence of a receptor, incorporated into the chimeric *trans*-activating regulatory factor, can be selected from a variety of receptor groups including but not limited to vitamin, steroid, thyroid, orphan hormone, retinoic acid, thyroxine, or GAL4 receptors. The chimeric *trans*-activating regulator factor is usually located within the sequence of the promoter. In one preferred embodiment the promoter used in the vector is the  $\alpha$ -actin promoter and the receptor is the vitamin D receptor.

"Receptor" as used herein includes natural receptors (i.e., as found in a cell *in vivo*) as well as anything that binds alike and causes compartmentalization changes in a cell.

Another embodiment of the regulatory system includes the construction of a vector with two functional units. One functional unit expresses a receptor. This functional unit contains elements required for expression including a promoter, a nucleic acid sequence coding for the receptor, and a 3' UTR and/or a 3' NCR. The second functional unit expresses GHRH or a derivative or RNA and contains, in addition, a

response element corresponding to the receptor, a promoter, a nucleic acid cassette, and a 3' UTR and/or a 3' NCR. These functional units can be in the same or separate vectors.

5       The first functional unit expresses the receptor. It is favorable to use a receptor not found in high levels in the target tissue. The receptor forms an interaction, e.g., ionic, non-ionic, hydrophobic, H-bonding, with the response element on the second  
10 functional unit prior to, concurrent with, or after the binding of the agent or ligand to the receptor. This interaction allows the regulation of the nucleic acid cassette expression. The receptor can be from the same nonlimiting group as disclosed above. Furthermore, the  
15 vector can be myogenic specific by using myogenic specific 3' UTR and/or 3' NCR sequences.

In an exemplary preferred embodiment the plasmid can be pSK-GHRH or a plasmid comprising a nucleotide sequence which is the same as the sequence of pSK-GHRH.  
20 This is only an example and is meant to be non-limiting. Thus, sequence changes or variations can be made to one or more of the sequence elements, such as the 5' and 3' flanking regions.

In this context, the word "same" means that the  
25 sequences are functionally equivalent and have a high degree of sequence identity. However, the sequences may have a low level of sequence differences, such as by substitution, deletion, or addition of one or more nucleotides. Such sequences will preferably be less  
30 than 10%, more preferably less than 5%, and still more preferably less than 1% of the total sequence.

In particular embodiments, the vectors of the above aspect may alternatively comprise, consist essentially

of, or consist of the stated elements or sequences.

A related aspect of the invention provides a formulation for delivery and expression of a GHRH gene in a cell, preferably a human GHRH gene. The  
5 formulation includes a vector of the above aspect together with one or more other components which can, for example, act to stabilize the vector or to enhance transfection efficiency, but can also provide other functions. In a preferred embodiment, the formulation  
10 includes the vector in a solution having between about 0.5% and 50% polyvinyl pyrrolidone (PVP), preferably about 5% PVP. Preferably, the PVP has a weight average molecular weight of about 50,000 g/mol. Further information is disclosed in PCT US95/17038. However,  
15 another example of a formulation includes the vector with a cationic lipid (e.g., as described in U.S. Patent 4,897,355, issued January 30, 1990), and can also include a co-lipid, such as a neutral co-lipid, e.g., cholesterol.

20 In reference to the formulations of this invention, the term "about" indicates that in preferred embodiments, the actual value for a particular parameter is in the range of 50%-200% of the stated value.

Another related aspect of the invention features a  
25 transgenic animal, at least some cells of which contain vectors of the first aspect of the present invention. These cells include germ or somatic cells. The transgenic animals can be used as models for studying human diseases, for assessing and exploring novel  
30 therapeutic methods, to assess the effects of chemical and physical carcinogens, and to study the effect of various genes and genetic regulatory elements. In addition, transgenic animals can be used to develop



commercially important livestock species.

A fourth related aspect of the present invention features cells transformed with a vector of the present invention for expression of a GHRH nucleic acid  
5 sequence, preferably a human hGHRH (hGHRH) nucleic acid sequence.

As used herein, "transformation" is the change in a cell's phenotypic characteristics by the action of a gene expressing a gene product. The gene causing the  
10 phenotypic characteristic change has been transfected into the cell.

The term "transfection" as used herein refers to a mechanism of gene transfer which involves the uptake of DNA by a cell or organism. Following entry into the  
15 cell, the transforming DNA may recombine with that of the host by physically integrating into the chromosomes of the host cell, may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. Preferably the  
20 transforming DNA does not integrate.

Transfection can be performed by *in vivo* techniques as described below, or by *ex vivo* techniques in which cells are co-transfected with a vector containing a selectable marker. This selectable marker is used to  
25 select those cells which have become transformed. It is well known to those skilled in the art the type of selectable markers to be used with transfection/transformation studies. An example of such a marker is a *neo* gene, providing neomycin/kanamycin resistance.

30 Transfection/transformation can be tissue-specific, i.e., involve the use of myogenic specific vectors which cause expression of the nucleic acid cassette predominantly in the tissue of choice. In particular,

tissue specificity can be directed to myogenic cells by using a promoter and/or 3'UTR and/or 3' NCR sequences specific for myogenic tissue expression.

A fifth related aspect of the present invention features methods for transfecting a cell with the vectors of the present invention. These methods comprise the steps of contacting a cell *in situ* with a vector of the present invention for sufficient time to transfect the cell. As discussed above, transfection can be *in vivo* or *ex vivo*.

A sixth related aspect of the invention provides a method for delivery and expression of a GHRH gene, preferably a hGHRH gene. The method comprises transfecting a plurality of cells with a vector of the first aspect and incubating the cells under conditions allowing expression of a nucleic acid sequence of the vector, which codes for GHRH. The "conditions allowing expression" may be any of a variety of conditions, including *in vivo* and *in vitro* conditions. Under such conditions, the cells will produce the gene product from the vector DNA in detectable quantities.

A seventh related aspect of the present invention features a method for treating a disease or condition by transfecting cells with the above-referenced vectors. Such disease or condition may, for example, be localized or systemic. These vectors contain nucleic acid sequences coding for growth hormone releasing hormone. Diseases and conditions can include but are not limited to burn, sepsis, trauma associated pathology, chronic obstructive pulmonary disease, aging associated osteoporosis, atherogenesis, atherosclerotic cardiovascular, cerebrovascular, or peripheral vascular disease, growth disorders and hemophilia.

The muscle atrophy to be treated may be due to any of a variety of different causes. For example, muscle weakness may be primarily due to disuse atrophy which commonly occurs in situations such as joint replacement, to muscle wasting during ageing, or to disease related cachexia. The causes may also include genetic causes of muscular atrophy, including, for example, muscular dystrophy. These causes and conditions are only exemplary and are not limiting to the invention.

Thus, "localized" disease or condition refers to those in which there is specific nerve or muscle damage or atrophy to a defined and limited area of the body. A specific example is disuse atrophy. A "systemic" disease or condition refers to those which relate to the entire organism, or is widely distributed at a number of locations within the body. Examples include growth disorders, neuropathies, and muscular dystrophy.

The methods of treating disease of the present invention feature methods for establishing expression of GHRH in tissue by administration of a vector. These methods of use of the above-referenced vectors comprise the steps of administering an effective amount of the vectors to a human, animal or tissue culture.

The term "administering" or "administration" as used herein refers to the route of introduction of a vector or carrier of DNA into the body. The vectors of the above methods and the methods discussed below may be administered by various routes. In particular a preferred target cell for treatment is the skeletal muscle cell.

The term "skeletal muscle" as used herein refers to those cells which comprise the bulk of the body's musculature, i.e., striated muscle.

Administration can be directly to a target tissue or may involve targeted delivery after systemic administration. The preferred embodiments are by direct injection into muscle or targeted uptake into muscle  
5 after intra-venous injection.

The term "delivery" refers to the process by which the vector comes into contact with the preferred target cell after administration. Administration may involve needle injection into cells, tissues, fluid spaces, or  
10 blood vessels, electroporation, transfection, hypospray, iontophoresis, particle bombardment, or transplantation of cells genetically modified *ex vivo*. Examples of administration include intravenous, intramuscular, aerosol, oral, topical, systemic, ocular,  
15 intraperitoneal and/or intrathecal.

The preferred means for administration of vectors described above involves the use of formulations for delivery to the target cell in which the vector is associated with elements such as lipids, proteins,  
20 carbohydrates, synthetic organic compounds, or inorganic compounds which enhance the entry of the vector into the nucleus of the target cell where gene expression may occur. A particular example is polyvinyl pyrrolidone (PVP).

25 The term "formulation" as used herein refers to non-genetic material combined with the vector in a solution, suspension, or colloid which enhances the delivery of the vector to a tissue, uptake by cells within the tissue, intracellular trafficking through the  
30 membrane, endosome or cytoplasm into the nucleus, the stability of the vector in extracellular or intracellular compartments, and/or expression of genetic material by the cell.

In a preferred embodiment of the present invention the vector and formulation comprises a nanoparticle which is administered as a suspension or colloid. The formulation can include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. Examples of elements which are included in a formulation are lipids capable of forming liposomes, cationic lipids, hydrophilic polymers, polycations (e.g. protamine, polybrine, spermidine, polylysine), peptide or synthetic ligand recognizing receptors on the surface of the target cells, peptide or synthetic ligand capable of inducing endosomal-lysis, peptide or synthetic ligand capable of targeting materials to the nucleus, gels, slow release matrices, salts, carbohydrates, nutrients, or soluble or insoluble particles as well as analogues or derivatives of such elements. This includes formulation elements enhancing the delivery, uptake, stability, and/or expression of genetic material into cells. This list is included for illustration only and is not intended to be limiting in any way.

Another embodiment of the present invention features the above vectors with coating elements that enhance expression as well as uptake by the cell. The term "coating" as used herein refers to elements, proteins or molecules used to associate with the vector in order to enhance cellular uptake. In particular, coating includes a DNA initiation complex and histones.

The coating improves the stability of the vector, its entry into the nucleus, and the efficiency of transcription.

The term "DNA initiation complex" as used herein refers to a complex containing a serum response factor, a transcription initiation factor and a trans-regulatory

factor. The serum response factor is attached to or interacts with the serum response element within the promoter region of the vector. The transcription initiation factor and the trans-regulatory factor then  
5 interact with the serum response factor and the promoter, in particular the TATA box within the promoter, to form a stable DNA complex. The term "histone" as used herein refers to nuclear proteins which associate with and/or bind to DNA, e.g., a vector.  
10 The histones can bind specifically or non-specifically to the DNA.

The term "effective amount" as used herein refers to sufficient vector administered to humans, animals or into tissue culture to produce the adequate levels of  
15 protein or RNA. One skilled in the art recognizes that the adequate level of protein or RNA will depend on the use of the particular vector. These levels will be different depending on the type of administration and treatment or vaccination.

20 The methods for treating diseases as disclosed herein includes treatment with biological products (specifically proteins as defined above) in which the disease being treated requires the protein to circulate through the body from the general circulation. For  
25 example, disorders which might be treated by the present invention include osteoporosis by expression of GHRH or its binding proteins. The selection of the appropriate protein to treat various diseases will be apparent to one skilled in the art.

30 In treating disease, the present invention provides a means for achieving: (1) sufficiently high levels of a particular protein to obtain a therapeutic effect; (2) controlled expression of product at levels which are

sufficient for therapeutic effect and lower than the toxic levels; (3) controlled expression in certain tissues in order to obtain reproducible pharmacokinetics and levels of gene expression; and (4) delivery using clinically and pharmaceutically acceptable means of administration and formulation rather than transplantation of genetically engineered and selected cells.

In doing so, the present invention provides significant advances over the art. First, promoters from viral genomes and viral vectors which were used to obtain high level expression in tissue, were not able to provide controlled expression. Second, promoters from various tissue-specific genes which were used to obtain controlled expression in transgenic animals and animal models of gene therapy did not have a sufficiently high level of expression to obtain therapeutic effect. In addition, in treating diseases with the present invention, the ability to raise antibodies against protein products does not reflect the ability to achieve controlled expression of proteins within the therapeutic range.

An eighth related aspect of the present invention features a method of gene replacement for inherited genetic diseases of muscle. This method includes the transfection of muscle cells with the above-referenced vectors.

The genetic material which is incorporated into the cells from the above vectors can be any natural or synthetic nucleic acid. For example, the nucleic acid can be: (1) not normally found in the tissue of the cells; (2) normally found in a specific tissue but not expressed at physiological significant levels; (3)

normally found in specific tissue and normally expressed at physiological desired levels; (4) any other nucleic acid which can be modified for expression in skeletal muscle cells; and (5) any combination of the above. In addition to the genetic material which is incorporated into tissue, the above reference is also applicable to genetic material which is incorporated into a cell.

By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

The present invention also concerns a gene therapy approach in which a species-specific GHRH cDNA plasmid based expression vector or other species-specific GHRH expression vectors are targeted into peripheral organs and expressed by the transfected cells. The species-specific GHRH polypeptide is then processed, secreted and transported to the anterior pituitary, where it



stimulates GH release.

As used herein, a "plasmid" is an extrachromosomal genetic element consisting of a circular duplex of DNA which can replicate independently of chromosomal DNA.

5 Plasmids are used in gene transfer, as the vehicle by means of which DNA fragments can be introduced into a host organism, and are associated with the transfer of antibiotic resistance.

The present invention concerns a method of  
10 expressing growth hormone releasing hormone (GHRH) in a non-human vertebrate animal comprising the step of inserting a DNA carrier vehicle containing a gene sequence encoding a growth hormone releasing hormone polypeptide sequence operatively linked to a vertebrate  
15 gene promoter into said non-human vertebrate animal tissue under conditions where said gene (a segment of DNA which codes for a specific polypeptide or RNA molecule) is expressed and produces hormone releasing hormone.

20 The term "non-human vertebrate animal" encompasses all animals having a backbone or spinal column, except for human beings. Vertebrate animals include fishes, amphibians, reptiles, birds and mammals.

As used herein, a "DNA carrier vehicle" refers to  
25 some means by which DNA fragments can be introduced into a host organism or host tissue. The DNA carrier vehicle may be designed to incorporate the gene of interest and any accessory genetic sequences.

The "gene sequence" preferably is a nucleic acid  
30 molecule.

The present invention concerns gene sequences that encode for a growth hormone releasing hormone having one of the following sequences; **SEQ ID NO.5**, **SEQ ID NO.6**,

SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, or SEQ ID NO.11;  
or where the non-human vertebrate animal is one of the  
following species; porcine, bovine, equine, canine,  
feline, caprine, avian (chicken, turkey, duck), ovine or  
5 fish.

The present invention also can involve GHRH gene  
sequence in the DNA carrier vehicle that contain no  
intervening sequences in the GHRH region.

A further object of the invention is use of a DNA  
10 carrier vehicle in which the promoter is from a skeletal  
 $\alpha$ -actin gene.

The present invention includes a DNA carrier  
vehicle which is injected into said animal muscle.

The present invention includes a plasmid DNA  
15 vector, adenovirus or adeno-associated virus as DNA  
carrier vehicles capable of infecting non-human  
vertebrate animals in various tissues.

The present invention can include a DNA carrier  
vehicle in which the promoter-GHRHcDNA-3'UTR is  
20 incorporated into an adeno-associated virus. The  
present invention can additionally include an embodiment  
where the vectors encode for an Arg-Arg sequence before  
a tyrosine or a histidine.

A further object of the invention is incorporation  
25 of a gene switch sequence into the DNA carrier vehicle.

The present invention also includes a gene sequence  
which is a chimeric synthetic cDNA encoding GHRH  
comprising a mouse specific fragment and a species  
specific fragment and wherein the mouse specific  
30 fragment contains the first 45 nucleotides and encodes  
the first 15 amino acids of the mouse GHRH, and said  
mouse specific fragment is fused in frame with a  
species-specific fragment containing 87 nucleotides

which encode the 16th to 44th amino acids of a species-specific GHRH. This chimeric sequence provides resistance against dipeptidases.

As used herein, a "chimera" refers to a molecule  
5 with genetic material from genetically different organisms.

Furthermore, the present invention can include a species-specific fragment for GHRH encoding a polypeptide from one of the following animal species;  
10 porcine, bovine, equine, canine, feline, caprine, avian (chicken, turkey, duck), ovine or fish or encoding for one of the following GHRH polypeptides; SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, or SEQ ID NO.11.

15 The present invention also concerns a method whereby skeletal muscle can be transfected *in vivo* by direct plasmid DNA injection or direct injection of other DNA carrier vehicles.

The present invention further concerns growth  
20 promoting myogenic expression plasmid vectors, pSK-GHRH, that drive high-level GHRH expression from animal muscle.

For example, species-specific GHRH can be secreted *in vitro*, in primary chicken and pig myotube cultures,  
25 and *in vivo*, after the intramuscular injection into regenerating quadriceps muscle of immunocompetent adult C57/B16 mice, or *in vivo* in the appropriate vertebrate species. Intramuscular injection of pSK-GHRH results in increased serum mGH, several fold over control values,  
30 for at least two weeks, and increased liver IGF-1 mRNA levels and enhances animal growth as compared to control animals.

The invention features a plasmid DNA based system which contains a vertebrate gene promoter that provides constitutive transcriptional activity. Other suitable vectors may also be used. The invention may also  
5 utilize muscle or other tissue specific promoters, or viral promoters active in animal cells. Human GHRH sequence is not desirable for use in other vertebrate species because it is antigenic and produces antibodies following injection into lower vertebrate animals. The  
10 DNA of the invention contains no intervening sequences anywhere in the plasmid DNA. The invention will use the target tissue's transcription, translation and secretory activities to transcribe the GHRH mRNA, correctly translate and then process the GHRH precursor protein,  
15 which, in turn, allows for secretion into the systemic blood supply. The increased levels of secreted GHRH will stimulate secretion of GH from the target animal's anterior pituitary.

Several embodiments of the invention involve GHRH  
20 expression for ectopic expression of a truncated GHRH from muscle, liver, heart, lung and vascular tissues by a plasmid DNA vector. This vector may contain eukaryotic promoters including various cell or tissue specific promoters (e.g., muscle, endothelial cell,  
25 liver), various viral promoters and enhancers, and various GHRH cDNAs isogenically specific for each animal species including porcine, equine, bovine, canine, feline, caprine, ovine, avian (chicken, turkey, duck) or fish. The vector may also contain a chimeric GHRH cDNA  
30 composed of the first 15 amino acids of the mouse GHRH following the processed N-terminal histidine, numbered 1, fused in frame with a specific animal GHRH species fragment covering amino acid 16 up to 44 amino acids, a

synthetic stop codon, an SV40 or a growth hormone 3' untranslated region containing polyadenylation sites. Any and all of these embodiments may utilize suitable vectors other than a plasmid DNA.

5           The plasmid of the invention may be incorporated into adenoviral and adenoviral-associated viruses and injected into muscle or into the blood system. DNA taken up into the cellular nuclei of tissue allows for transcription of a messenger RNA encoding a truncated  
10 GHRH polypeptide which is then translated into a precursor GHRH protein. The precursor protein requires metalloprotease or other processing to allow for a biologically active GHRH to be secreted. The precursor protein is trimmed to either a N-terminal tyrosine or a  
15 N-terminal histidine depending upon the animal GHRH species. Ectopic secretion from muscle and other tissues including liver, pancreas, kidney and heart of the correctly processed GHRH into the blood system, increases the concentration of GHRH in the blood, which  
20 then causes a profound stimulation of growth hormone (GH) secretion from the anterior pituitary of the target animal. Skeletal muscle-secreted GHRH is biologically active, as demonstrated by eliciting robust GH release following a single intramuscular injection of 100  $\mu$   
25 plasmid CMV-GHRH DNA sufficient to elevate GH levels 3 to 4 fold for up to 2 weeks, to enhance liver IFG-1 gene expression and to increase body weight approximately 10%. Thus, plasmid based-GHRH can serve as a potent GH secretagogue in animals.

30           One embodiment of the invention includes a novel plasmid vector which is capable of directing high-level gene expression in a skeletal muscle specific manner. A 228bp fragment of hGHRH, which encodes for the 31 amino

acid signal peptide and the entire mature peptide hGHRH(1-44)OH(Tyr1-Leu44) is cloned into a pBS-derived vector. Gene expression is controlled by a 448bp fragment (-424 / +24) of the avian skeletal  $\alpha$ -actin  
5 gene, which contains several evolutionarily conserved regulatory elements that accurately initiate skeletal  $\alpha$ -actin transcripts and drives transcription of a variety of reporter genes specifically in differentiated skeletal muscle cells. The GHRH coding region is  
10 followed by the 3' untranslated region of human growth hormone cDNA.

In another embodiment the cytomegalovirus promoter and enhancer is used. In a preferred embodiment the promoter is linked to a synthetic GHRH cDNA which  
15 contains any non-muscle 3' untranslated region cDNA. In one preferred embodiment a bovine growth hormone 3' untranslated region cDNA is used.

In one embodiment of the invention the plasmid DNA is injected into muscle. In yet another preferred  
20 embodiment the promoter GHRHcDNA-3'UTR can be incorporated into a virus, such as an adeno-associated virus for viral infection of muscle.

In another embodiment the invention is incorporated in adenoviruses and allowed to infect a variety of  
25 tissues that will then express species specific GHRH mRNA in any tissue the adenovirus infects.

In another embodiment the GHRH vectors are made with species specific GHRH that contains a metalloenzyme processing sequence Arginine-Arginine before a Tyrosine  
30 or a Histidine. Each GHRH polypeptide secreted can be made isogenic so that it is identical to the actual animals' GHRH.

Another embodiment of the invention employs a gene

switch element in the DNA carrier vehicle.

In another embodiment a chimera synthetic cDNA encoding GHRH is used that contains the first 45 nucleotides to encode the first 15 amino acids of the mouse GHRH fused in frame with the nucleic acid sequence encoding the 16th to the 44th amino acids of the species-specific GHRH cDNA sequence. This chimeric sequence is effective in providing resistance against proteolytic degradation by dipeptidases.

In other embodiments of the invention the animal species of GHRH encoded for may include: porcine, bovine, equine, canine, feline, caprine, avian (chicken, turkey, duck), ovine and fish.

Other embodiments of the invention utilize nucleotides sequences coding for one of the GHRH polypeptides expressed by any one of SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, and SEQ ID NO.11. A skilled artisan will readily recognize that these polypeptides can be encoded for by a number of nucleotide sequences.

Other features and advantages of the invention will be apparent from the following detailed description of the invention in conjunction with the accompanying drawings and from the claims.

25

### **Brief Description of the Drawings**

**Figure 1** is a schematic drawing of the chicken skeletal  $\alpha$ -actin gene which includes the location of certain unique restriction sites.

**Figure 2** is a schematic representation of a myogenic vector system.

**Figure 3** is a schematic representation of the

exemplary plasmid pSK-GHRH.

**Figure 4** is a chart showing the codon usage frequencies for highly expressed human genes.

**Figure 5** shows the sequences of wild-type GHRH (SEQ ID NO. 1) and a synthetic sequence having optimal codon usage and encoding GHRH (SEQ ID NO. 2).

**Figure 6** shows the nucleotide sequence of a NotI/SalI fragment of the pSK-GHRH plasmid which surrounds the GHRH cDNA insert. The portion coding for GHRH is in bold.

**Figure 7** shows the level of human GHRH secreted in the culture media of transfected chicken primary myoblasts as measured by specific RIA: pSK-GHRH (black, n = 8), compared with pSK-LacZ (white, n=8). The results are presented as means  $\pm$  s.e.m (\*, p value is  $\leq$  0.002).

**Figure 8** is the experimental design for stimulation of the GH secretion by hGHRH secreted by primary pig myoblasts (ppm) transfected with pSK-GHRH.

**Figure 9** shows pig GH release in primary pig anterior pituitary culture after 24h challenge in response to: 10ng hGHRH (white, n = 4) = long of synthetic hGHRH(1-44)NH<sub>2</sub> have been mixed in the ppm culture media; pSK-GHRH (black, n = 4) = the conditioned culture media from 1 million ppm transiently transfected with pSK-GHRH; pSK-LacZ (gray, n = 4) = the conditioned culture media from 1 million ppm transiently transfected with pSK-LacZ. The results are presented as means  $\pm$  s.e.m (\*, p value for pSK-GHRH  $\leq$  0.002).

**Figure 10** shows expression of pSK-GHRH assessed by RT-PCR at 3-21 days after i.m. injection of 100  $\mu$ g pSK--GHRH in the regenerating left quadriceps muscle of adult



C57/B16 mice.

**Figure 11** shows body weight at different time points after a single injection in the regenerating left quadriceps muscle of adult mice of pSK-GHRH (black, n = 5 6), compared to that of age-matched pSK-LacZ injected animals (white, n = 6). The results are presented as means  $\pm$  s.e.m. Significant difference \*,  $p \leq 0.05$  and  $p \leq 0.03$ , was observed at day 14 and day 21, respectively.

**Figure 12** shows a Northern blot analysis of chicken 10 primary myoblast culture transiently transfected with pSK-GHRH or pSK-LacZ, as a control. 10  $\mu$  of total RNA were separated, transferred onto a nylon membrane and hybridized with a hGHRH cDNA probe and then with a mouse GAPDH probe in order to normalize the results.

15 **Figure 13** shows expression of pSK-GHRH assessed by RT-PCR at 3-21 days after i.m. injection of 100  $\mu$  pSK-GHRH into the regenerating left quadriceps muscle of adult C57/B16 mice. RT-PCR reaction from 1  $\mu$  of total RNA - upper panel: 254 bp PCR fragment using SK-GHRH 20 cDNA specific oligonucleotides; lower panel: 497bp PCR fragment using mouse cytoskeletal  $\beta$ -actin cDNA specific oligonucleotides, in pSK-GHRH injected animals or pSK-LacZ injected animals.

**Figure 14** shows mouse serum growth hormone was 25 measured by rat GH heterologous radioimmunoassay after i.m. injection of 100  $\mu$  pSK-GHRH in adult C57/B16 mice.

Control sera were obtained from mice injected with pSK-LacZ. The results are presented as means  $\pm$  s.e.m. Significant differences of \*,  $p \leq 0.03$  and  $p \leq 0.05$  were 30 obtained at day 7 and day 10, respectively.

**Figure 15** shows Northern blot analysis of mouse liver RNA in pSK-GHRH (+) or pSK-LacZ (-) injected

animals. The animals were killed and livers harvested at day 3-21, 20  $\mu$  of total RNA was separated, transferred onto a nylon membrane and hybridized with an mIGF-1 cDNA probe and then a mouse 18S probe in order to  
5 normalize the results.

**Figure 16** shows increased gain in body weight after a single injection of pSK-GHRH in the regenerating left quadriceps muscle of adult mice. pSK-GHRH (black, n = 6) are compared to that of age-matched pSK-LacZ injected  
10 animals (white, n = 6). The results are presented as means  $\pm$  s.e.m. \*  $p \leq 0.05$  and  $p \leq 0.03$ , was observed at day 14 and day 21, respectively.

**Figure 17** shows generation of mouse anti human GHRH antibodies following intramuscular injection of pSK-GHRH  
15 in C57/B16 mice. 21 (n=3) and 28 (n=3) days after injection of pSK-GHRH and 21 days after pSK-LacZ (n=3) injections, serial dilutions of sera were assayed for anti-GHRH antibodies by ELISA. A significant difference,  $p \leq 0.05$  was observed between pSK-GHRH  
20 injected mice and control curves.

The drawings are not necessarily to scale, and certain features of the invention may be exaggerated in scale and shown in schematic form in the interest of clarity and conciseness.

25

#### **Description of the Preferred Embodiments**

GHRH administration represents a more physiological  
30 alternative of increasing subnormal GH and IGF-I levels (Corpas et al., 1993, *J. Clin. Endocrin. & Metab.* 76:134-138). Even though in mammalian species GH secretion is pulsatile as a result of a complex

neuroendocrine regulation process, (Betherrat et al., 1995, *Eur. J. Endocrin.* 132:12-24) numerous studies have shown that continuous infusion of GHRH augments GH secretion in normal adults and GH deficient children without evidence of desensitization of the somatotrophs and promotes growth in short, slowly growing children (Low, 1993, *J. Pediatric Endocrin.* 6:15-20). Current limitations of recombinant GHRH therapy are the high cost of recombinant proteins, the short half-life of the peptides *in vivo* and the requirement for frequent administration (1-3 times/day) given as subcutaneous or intravenous injections.

The human GHRH genetic locus, as well as the gene and cDNA have been characterized (Mayo et al., 1985, *Proc. Natl. Acad. Sci. USA* 82:63-87; Mayo et al., 1983, *Nature* 306:86-88. The gene includes 5 exons and spans 10 kb on human chromosome 20 (Riddell et al., 1985, *Genetics* 11:189-195). Biological activity requires post-translational processing of the GHRH precursor protein. The preprohormones are proteolytically clipped to two mature protein species: (1-40)GHRH with a carboxy terminal group and (1-44)GHRH with a carboxy terminal amide group. Part of exon 2, all of exon 3 and part of exon 4 encode for the 31 amino acids signal peptide and the entire mature peptide (Tyr1→Leu44).

Somatic gene therapy can provide an alternative treatment for growth disorders, catabolic conditions and for the general reduction of GH in the elderly. It is well established that ectopically secreted GHRH, as mature peptide or truncated molecules (as seen with pancreatic islet cell tumors and various located carcinoids) are often biologically active and can even produce acromegaly (Esch et al., 1982), *Biochem. &*

*Biophys. Res. Comm.* 109:152-158; Thorner et al., 1984, *J. Clin. Endocrin. & Metab.* 59:846-849. In a gene therapy approach, the human GHRH cDNA can be targeted into peripheral organs, expressed by the transfected  
5 cells and the peptide processed, secreted, transported to the anterior pituitary, where it can stimulate GH release.

Skeletal muscle can be transfected *in vivo* by direct plasmid DNA injection and an encoded gene can be  
10 expressed at significant levels for periods of time, up to about 19 months (Wolff et al., 1992, *Human Mol. Genet.* 1:363-369; Wolff et al, 1990, *Science* 247:1465-1468; Davis et al, 1993, *Human Gene Therapy* 4:151-159; Walls, 1993, *FEBS Lett.* 332:170-182). A limitation of  
15 this approach is the relatively low efficiency of gene transfer into nonregenerating adult muscle, though the transfer efficiency can be enhanced by treating the target muscle 3-7 days prior to plasmid DNA injection with 0.75% bupivacaine.

20 The vectors and methods of this invention provide for the delivery and expression of GHRH in mammalian cells, e.g., in human cells. It has been shown that IGF-I plays an important role in normal muscle development, muscle growth and hypertrophy, muscle  
25 regeneration and maintenance/regeneration of peripheral nerves. GHRH increases the levels of GH and IGF-I. Thus, delivery and expression of GHRH from an expression vector is expected to modulate these process.

The following are specific examples of preferred  
30 embodiments of the present invention and are not intended to limit the invention. These examples demonstrate how the expression vector systems of the present invention can be used in construction of various

cellular or animal models, and how genes can be regulated by sequences within such vectors. The description and utility of such vectors and related vectors is discussed herein and is amplified upon in  
5 Schwartz et al., U.S. Patent No. 5,298,422, entitled "Myogenic Vector Systems," and co-pending application Schwartz et al., Application No. 08/472,809, entitled "Expression Vector Systems and Method of Use", which are hereby specifically incorporated by reference herein,  
10 including drawings. Such vectors can incorporate nucleic acid sequences encoding GHRH and can be used for delivery and expression of GHRH.

Below are provided examples of specific regions of 5' UTR and 3' UTR and/or 3' NCR regions of myogenic  
15 genes that can be used to provide certain functionalities to an expression vector, and thus within a transformed cell or animal containing such a vector. Those in the art will recognize that specific portions of these regions can be identified as that containing  
20 the functional nucleic acid sequence providing the desirable property, and such regions can be readily defined using routine deletion or mutagenic techniques or their equivalent. Such regions include the promoter, enhancer and *cis*- and *trans*-acting elements of a  
25 regulatable system. As noted herein, such controlling segments of nucleic acid may be inserted at any location on the vector, although there may be preferable sites as described herein.

### 30 Isolation of Chicken Skeletal $\alpha$ -Actin Gene

The nucleic acid sequence of the skeletal  $\alpha$ -actin gene has been characterized in chicken, rat, mouse and human. Fornwald et al, 1982, *Nucl. Acids Res.* 10:3861-

3876; R. Zakut, 1982, *Nature* 298:857-859; French et al, 1990, *Gene (Amst.)* 88:173-180; Hu et al, 1986, *Mol. Cell. Biol.* 6:15-25; Minty et al, 1986, *Mol. Cell. Biol.* 6:2137-2148. The skeletal  $\alpha$ -actin gene is a member of  
5 the actin multigene family, which, in vertebrates, is made up of three distinct classes of actin isoforms termed as "cytoplasmic", "smooth muscle", and "striated" on the basis of their cellular distribution and pattern of expression in adult tissues. The striated actins,  $\alpha$ -  
10 cardiac and  $\alpha$ -skeletal, are co-expressed specifically in cardiac myocytes and skeletal myofibers. Expression of the  $\alpha$ -cardiac and  $\alpha$ -skeletal actin genes is sequentially up-regulated in developing cardiac and skeletal muscle with the skeletal isoform predominating in adult  
15 skeletal muscle. (Vandekerckhove & Weber, 1984, *J. Mol. Biol.* 179:391-413; McHugh et al., 1991, *Dev. Biol.* 148:442-458; Hayward & Schwartz, 1986, *J. Cell Biol.* 102:1485-1493.) The chicken skeletal  $\alpha$ -actin gene is the most highly expressed gene in adult chicken skeletal  
20 muscle comprising approximately 8% of the poly(A) RNA.

Numerous experiments *in vitro* and *in vivo* have established that the regulatory sequences which confer cell type restricted and developmentally regulated expression to the skeletal  $\alpha$ -actin gene are primarily  
25 concentrated in the immediate 5' promoter region. (Bergsma et al., 1986, *Mol. Cell. Biol.* 6: 2462-2475; Taylor et al., 1988, *Genomics*. 3(4): 323-36; Petropoulos et al., 1989, *Mol. Cell. Biol.* 9:3785-3792; Carson et al., 1995, *Am. J. Physiol.* 268:C918-24.)

30 These regulatory sequences are highly conserved in the promoter regions of all of the known vertebrate skeletal  $\alpha$ -actin genes from aves to man. Regulatory

sequences derived from the chicken skeletal  $\alpha$ -actin gene were utilized in construction of the GHRH expression cassette, though other embodiments can utilize other actin or  $\alpha$ -skeletal actin genes.

5           The primary sequences of the skeletal  $\alpha$ -actin genes of the various species were deduced from overlapping cDNA clones. To obtain full genes, the cDNA clones were used to screen genomic DNA. For example, the 25 Kb EcoRI fragment of chicken genomic DNA isolated from a  
10   lambda Charon 4A vector, contains the 6.2 Kb skeletal  $\alpha$ -actin gene on a single HindIII site of pBR322 is shown in **Figure 1**. Chang et al., *Mol. Cell. Biol.* 4:2498-2508 (1984). Nuclear transcription runoffs were used to map the transcriptional domain of the skeletal  $\alpha$ -actin gene.

15   The chicken skeletal  $\alpha$ -actin control sequences have also been characterized (Bergsma et al., 1986, *Mol. Cell. Biol.* 6:2462-2475). DNA probes which encompassed portions of the 5' noncoding, promoter coding, and the contiguous 3' noncoding regions were cloned into M13  
20   vectors which provided sense and antisense probes. Nuclei isolated from fibroblasts, myoblasts and day 19 embryonic muscle cells were used in *in vitro* transcription assays to extend RNA transcripts with radioactive tagged nucleotides. Labeled RNA hybridized  
25   to dotted DNA probes showed that transcription terminates approximately 1 kb downstream of the skeletal  $\alpha$ -actin gene's poly A addition site. This is within a 800 bp PvuII fragment between +2800 and +3600 nucleotides from the start of transcription.

30           The 3' UTR and/or 3' NCR can be isolated by restriction endonuclease digestion of the 6.2 Kb actin gene with blunt cutter NaeI, which cuts 30 bp upstream

of the translation termination codon TAA. HindIII releases the 3' most portion of the actin gene from the vector pBR322 (**Figure 2**). The 3'UTR and 3'NCR were used to prepare DNA constructs. The skeletal  $\alpha$ -actin promoter and DNA flanking sequences (at least 411 nucleotides from the mRNA cap site) and DNA sequences extending through the skeletal 5' noncoding leader, first intron and up to the initiation of translation ATG, converted to a NcoI cloning site at +196, was liberated from a M13 double stranded DNA by XbaI and NcoI digestion, Klenow filled in and then linked into the XbaI and blunt SmaI sites of pBluescript II KS. The NcoI site is regenerated by this cloning step.

For certain vectors described in Schwartz et al., Application No. 08/472,809, the 3'UTR and 3'NCR on the 2.3 kb NaeI/HindIII fragment were directionally cloned into a blunt EcoRV site and the adjacent HindIII site of the pBluescript II KS vector cassette. The EcoRV and NaeI sites are destroyed. The restored NcoI site was used to insert cDNA sequences encoding polypeptides. Another cloning vector was constructed by inserting the skeletal  $\alpha$ -actin promoter from -411 to -11 adjacent to the 3'UTR and 3'NCR. This expression vector eliminates the first intron and the skeletal actin 5' leader sequence. These two vectors were used in preparing DNA constructs to test the efficacy of the 3'UTR and 3' NCR.

Results obtained using vectors having a skeletal  $\alpha$ -actin/GHRH/GH expression cassette are described below, illustrating the intracellular expression of GHRH from vector constructs and certain results of such expression.



### Expression Vector Construction Containing Human GHRH Gene

Constructions containing the skeletal  $\alpha$ -actin promoter were linked to the human GHRH cDNA (SEQ ID NO.

5 1) by standard recombinant DNA techniques as known in the art. Examples of a generalized expression vector structure utilizing skeletal  $\alpha$ -actin 5' and 3' sequences is shown in **Figure 2**.

The GHRH construction can be made so that a poly A addition site, e.g., the poly A site of GHRH, was linked to the 3'UTR of the GH gene. The sequence was added to increase the stability of nuclear GHRH RNA transcripts.

The poly A skeletal  $\alpha$ -actin 3' UTR can also be used in the construction. In this way GHRH RNA transcripts containing the skeletal  $\alpha$ -actin 3' UTR are stabilized and accumulate in skeletal muscle cells. In addition, by providing contiguous 3' NCR, GHRH is buffered against outside genomic sequences and is thus more protected from position effects, when integrated into the genome.

20 In addition, by providing natural terminating sequences, the additional regulatory sequences that mark the transcriptional domain of skeletal  $\alpha$ -actin prevent read through transcription, improve tissue specificity, developmental timing and transcriptional activity.

25 Presence of 3'NCR sequence allows for a single copy of the integrated vector to produce 40-100% of the transcriptional activity of the endogenous sequences.

The exemplary plasmid vector, pSK-GHRH was constructed using pIG0100A and additional constructs (pOGH and pVC0289A). A schematic representation of pSK-GHRH is shown in **Figure 3**. The pSK-GHRH expression plasmid contains a hGHRH gene expression cassette in a

plasmid backbone containing a kanamycin-resistance (KanR) gene. The plasmid backbone is as described for pIG0552 in co-pending United States provisional application 60/031,539, Coleman et al., entitled IGF-1  
5 EXPRESSION SYSTEM AND METHODS OF USE, filed December 2, 1996. The hGHRH gene expression cassette of pSK-GHRH contains: 1) a promoter derived from the chicken skeletal  $\alpha$ -actin promoter and first intron, 2) the human Growth Hormone Releasing Hormone (hGHRH) cDNA, and 3) a  
10 3' UTR/poly(A) signal from the human Growth Hormone (hGH) 3' untranslated region (3' UTR). The plasmid backbone is derived from pBluescript KS+ (Stratagene) with 1) the substitution of a kanamycin-resistance gene (*neo*) and prokaryotic promoter (pNEO, Pharmacia) in  
15 place of the ampicillin-resistance gene (*bla*) and 2) the deletion of the *fl* origin of replication.

The actual construction of pSK-GHRH primarily involved three starting plasmids, pIG0100A, pOGH and pVC0289A.

20 The chicken skeletal  $\alpha$ -actin promoter and first intron were obtained from plasmid pIG0100A (R. Schwartz, Baylor College of Medicine). The hGH 3' UTR was obtained from plasmid pOGH (Nicholas Institute, CA, USA). The hGHRH cDNA 228 bp fragment (part of exon 2,  
25 all exon 3 and part of exon coding for the 31 amino acid signal peptide and entire mature hGHRH1-44 peptide Tyr1-Leu44) was utilized. pIG0100A contains the chicken skeletal  $\alpha$ -actin promoter and first intron, human hIGF-1 cDNA, and chicken skeletal  $\alpha$ -actin 3' untranslated  
30 region and 3' flanking sequence in pBluescript KS+. As indicated above, the plasmid backbone, pVC0289A, includes the kanamycin-resistance gene, pUC origin of

replication, and a multicloning site.

The construction scheme used to produce pSK-GHRH from pIG0100A, pOGH, and pVC0289A incorporated the following steps. In order to construct hybrid pSK-GHRH, a 448 bp fragment (-424/+24) of avian skeletal  $\alpha$ -actin promoter (SK) was used (Lee et al., 1994, *J. Oncogene* 9:1047-1052; Chow et al., 1991, *PNAS* 88:1301-1305). The hGHRH cDNA 228 bp fragment (part of exon 2, all exon 3 and part of exon 4) coding for the 31 aminoacid signal peptide and entire mature hGHRH1-44 peptide (Tyr1→Leu44) has been cloned into BamHI / HindIII sites of pVC0289. The 3' untranslated region of hGH cDNA SmaI blunted/EcoRI 622bp fragment was cut from the commercial pOGH plasmid (Nichols Institute, CA, USA) and cloned into ClaI blunted/EcoRI sites of pVC0 289. The sequence of a NotI/SalI fragment of the plasmid, which includes the SK promoter, GHRH cDNA, and hGH 3' region is shown in **Fig. 6**.

The GHRH cDNA and plasmid sequences described herein are believed to be correct, however, the possible presence of a small percentage of nucleotide sequence errors will not impair the use of this invention. Those skilled in the art will understand how to obtain sequences coding for GHRH, e.g., hRHRH, such as by isolating a GHRH cDNA from a cDNA library using a probe or probes derived from the published GHRH sequence or from the sequence described herein. Such a sequence can be sequenced by routine methods to confirm or obtain the correct GHRH coding sequence. The 5' UTR and 3' UTR sequences reported herein can likewise be obtained by routine methods. Based on the present disclosure, those skilled in the art will also understand how to construct a vector containing a sequence encoding GHRH, which can

be used for delivery and expression of the GHRH *in vivo*.

Also, in addition to sequences encoding natural GHRH, e.g., the sequence encoding GHRH (1-44), other sequences encoding functional GHRH derivatives can be used, such as a sequence encoding hGHRH (1-40), or modified sequences which encode a GHRH derivative differing in one or a few amino acids but which retains the GH and IGF-1 related functions of native GHRH. Preferably, the encoded GHRH derivative will differ from a native GHRH, e.g., hGHRH (1-44) or hGHRH (1-40), by the addition, deletion, substitution, or a combination of these changes at a small number of amino acid residues. A small number is preferably 5 or fewer, more 3 or fewer, still more preferably 2 or fewer, and most preferably is one amino acid.

In addition to sequences encoding native length GHRH or a functional derivative, a coding sequence can also be used which encodes a prohormone which can be proteolytically cleaved to produce an active GHRH molecule or derivative. Such sequences are exemplified by natural hGHRH coding sequences which encode a polypeptide sequence which is cleaved to produce both hGHRH (1-44) and hRHRH (1-40) mature polypeptides.

In the pSK-LacZ construct, the  $\beta$ -galactosidase gene of *Escherichia coli*, with a nuclear localization signal (nls), is driven by the same SK promoter, but contains the 3' UTR of skeletal  $\alpha$ -actin gene (French et al., 1990, *Gene* 88:173-180).

Instead of the natural sequence coding for GHRH, it is advantageous to utilize synthetic sequences which encode GHRH. Such synthetic sequences have alternate codon usage from the natural sequence, and thus have

dramatically different nucleotide sequences from the natural sequence. In particular, synthetic sequences can be used which have codon usage at least partially optimized for expression in a human. The natural  
5 sequences do not have such optimal codon usage. Preferably, substantially all the codons are optimized.

Optimal codon usage in humans is indicated by codon usage frequencies for highly expressed human genes, as shown in **Fig. 4**. The codon usage chart is from the  
10 program "Human\_High.cod" from the Wisconsin Sequence Analysis Package, Version 8.1, Genetics Computer Group, Madison, WI. The codons which are most frequently used in highly expressed human genes are presumptively the optimal codons for expression in human host cells, and  
15 thus form the basis for constructing a synthetic coding sequence.

However, rather than a sequence having fully optimized codon usage, it may be desirable to utilize an GHRH encoding sequence which has optimized codon usage  
20 except in areas where the same amino acid is too close together or abundant to make uniform codon usage optimal.

In addition, other synthetic or derivative sequences can be used which have substantial portions of the codon usage optimized, for example, with at least  
25 50%, 70%, 80% or 90% optimized codons. Other particular synthetic sequences for GHRH can be selected by reference to the codon usage chart in **Fig. 4**. A sequence is selected by choosing a codon for each of the  
30 amino acids of the polypeptide sequences. DNA molecules corresponding to each of the polypeptides can then be constructed by routine chemical synthesis methods. For example, shorter oligonucleotides can be synthesized,

and then ligated in the appropriate relationships to construct the full-length coding sequences.

A particular preferred synthetic GHRH coding sequence is provided in **SEQ ID NO. 2**.

5

#### Myogenic Cell Cultures and DNA Transfer

Minimal Essential Medium (MEM), horse serum, gentamycin, Hank's Balanced Salt Solution (HBSS), lipofectamine were obtained from Gibco BRL, NY, USA.

10 Primary chicken myoblast culture was obtained as described (Bergame et al., *J. Molec. & Cell. Biol.* 6:2462-2475). The cells were plated 24h prior to transfection at a density of 1.5 million cells / 100 mm plate, in MEM supplemented with 10% horse serum (HIHS) ,  
15 5% chicken extract (CE) and gentamycin. Cells were maintained in a humidified 5% CO<sub>2</sub> 95% air atmosphere at 37°C.

Cells were transfected with 4µg of pSK-GHRH or pSK-LacZ per plate using lipofectamine, according to the  
20 manufacturer instructions. After transfection, the cells were changed in MEM, 2% HIHS, 2% CE for at least 24h to allow differentiation.

Primary pig myoblasts culture was obtained as described (Doumit & Merkel, 1992, *Tissue & Cell* 24:253-  
25 262). The cells were plated at a density of 1 million cells / 100mm plate and maintained in growth media for 2-3 days. The cells were passed for 2 times prior to transfection. The transfection and differentiation was made in the same conditions as for the primary chicken  
30 myoblast culture. The media and cells were harvested for analysis 48, 72 and 96h postdifferentiation in both cases. The efficiency of transfection estimated by β-galactosidase histochemistry on control plates was 10%.

One day before harvesting, cells were washed twice in HBSS and changed in MEM, 0.1% BSA. The collected media was conditioned by adding ¼ volumes of 1% trifluoroacetic acid (TFA) and 0.001% phenylmethanesulfonylfluoride (PMSF), frozen at -80°C, lyophilized, purified on C-18 Sep-Columns (Peninsula Laboratories, CA, USA), rehydrated and used in RIA or resuspended in media conditioned for primary pig anterior pituitary culture. The pig anterior pituitary culture was obtained by Dr. Thomas H. Welsh Jr. in the Department of Animal Sciences at Texas A&M University as described (Tanner et al., 1990, *J. Endocrinol.* 125:109-115). Pig GH was assayed as described (Barb et al., 1991, *Domestic Animal Endocrinology* 8:117-127). The samples and controls were assayed as described in quadruplicate. The cells were homogenized directly into Ultraspec RNA reagent (Biotechx Laboratories, TX, USA) for the isolation of total RNA.

20 Measurement of Secreted Levels of GHRH from GHRH Gene Delivery by the Expression Vector

A. *In vitro* expression of pSK-GHRH. We characterized a novel plasmid vector able to express in a skeletal muscle specific manner a high level of a target protein, hGHRH. A 228 bp fragment of hGHRH (part of exon 2, all exon 3 and part of exon 4), which encode for the 31 aminoacid signal peptide and the entire mature peptide hGHRH(1-44)OH (Tyr1-Leu44) (Mayo et al., 1985, *PNAS* 82:63-67) was cloned into a pBS-derived vector. The coding sequence was controlled by a 448 bp fragment (-424 / +24) of the avian skeletal  $\alpha$ -actin gene, which contain several evolutionarily conserved

regulatory elements that accurately initiate skeletal  $\alpha$ -actin transcripts and drive transcription of a variety of reporter genes specifically in differentiated skeletal muscle cells (Bergame et al, 1986, *J. Mol. & Cell. Biol.* 6:2462-2475; Chow et al., 1990, *J. Mol. & Cell. Biol.* 10:528-538; Lee et al., 1994, *J. Oncogene* 9:1047-1052). The coding region was followed by the 3' untranslated region of human growth hormone cDNA.

*In vitro* expression of pSK-GHRH was first examined in transiently transfected chicken primary myoblasts. The pSK-GHRH transfected cells and the controls, transfected with pSK-LacZ were placed into differentiation media for 24-72 h to initiate withdrawal from the cell cycle and induce post-fusion differentiation, then changed into a minimal serum-free media for a 24h pulse. Cells were harvested 48 to 96h post-differentiation. Northern blot analysis of cellular extracted RNA treated with DNase, showed the expected size transcripts of 0.35 kb, in myoblasts transfected with pSK-GHRH, but not in pSK-LacZ transfected myoblasts. The expression of pSK-GHRH peaked at 48h postdifferentiation and was reduced thereafter in comparison to the glycolytic enzyme GAPDH.

This pattern of activation is characteristic of the promoter utilized, which induces high levels of transgene expression in myotubes but not in replicating myoblasts (Bergame et al, 1986, *J. Mol. & Cell. Biol.* 6:2462-2475; Chow et al., 1990, *J. Mol. & Cell. Biol.* 10:528-538; Lee et al., 1994, *J. Oncogene* 9:1047-1052).

Conditioned media from pSK-GHRH and pSK-LacZ transfected myoblasts were harvested and purified on C18 Sep-Columns (which served two purposes: to separate the



peptide to be assayed from potentially interfering substances and to concentrate the samples) to determine levels of radioimmunoassayable hGHRH. Chicken primary myoblasts transfected with pSK-GHRH produced  
5 approximately 1.7 ng hGHRH(1-44)OH / million cells / h. Media from pSK-LacZ control transfected cells did not contain hGHRH higher than the untransfected controls (**Figure 7**). The decrease in hGHRH secreted into the media at 96h correlated with the decrease of mRNA  
10 between the 72 and 96h time-points. We concluded that the skeletal fibers transfected with pSK-GHRH are expressing and secreting at a high level hGHRH (1-44)OH.

B. *In vitro* activity of pSK-GHRH. Since anterior pituitary is the natural target of GHRH stimulation, we  
15 determined the biological activity of secreted GHRH from the media of pSK-GHRH transfected primary pig myoblasts (**Figure 8**). The *in vitro* potency of the hGHRH (1-44)OH molecule secreted by primary pig myoblasts (ppm) transiently transfected with pSK-GHRH was compared to  
20 that of the hGHRH (1-44)NH<sub>2</sub> synthetic molecule for the ability to stimulate GH release in primary pig anterior pituitary cells after a 24h stimulation at 37°C. GH release from the primary pig anterior pituitary cells rose from values of  $7 \pm 2$  ng / ml to  $82.5 \pm 3.1$  ng / ml  
25 ( $p \leq 0.002$ ) when stimulated with the culture media from 1 million primary pig myoblasts transiently transfected with pSK-GHRH. This value equals 72% of that obtained when the pituitary cells were stimulated with myoblast serum-free media mixed initially with 10 ng synthetic  
30 hGHRH (1-44)NH<sub>2</sub> then purified and processed as the test media (GH release in this case was  $115 \pm 3.2$  ng / ml) (**Figure 9**).

Thus, hGHRH(1-44)OH secreted by skeletal pig

myocytes transfected with pSK-GHRH retains functional activity in pig pituitary cell culture and induces secretion of physiologically significant levels of GH. This is an important finding, because the pig (1-44)GHRH, as well as the human molecule, is amidated, while the molecule which is expressed by our construct is non-amidated at amino acid 44. It has been shown in previous studies using synthetic molecules that hGHRH(1-44)OH is 30% less effective in releasing GH than the (1-44) amidated form (Ling et al, 1984, *Biochem. & Biophys. Res. Comm.* 123:854-861).

#### Insertion of Expression Vectors into Transgenic Mice

Transgenic mice carrying GHRH containing vectors can be generated by standard methods, e.g., by standard oocyte injection (Brinster, et al, *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985)) and bred to demonstrate stable transmission of transgenes to subsequent generations. Transgenics can be identified by polymerase chain reaction or Southern genomic DNA blotting analysis from tail cut DNA. Transgenics can be tested for muscle specific expression of the transferred GHRH vector by RNA blotting of total RNA isolated from several tissues.

25

#### Somatic Gene Transfer to Skeletal Muscle *in vivo*

*In vivo* expression and activity of pSK-GHRH. In addition to the *in vitro* determinations of GHRH expression from chicken primary myoblasts and the induction of GH secretion from pig primary anterior pituitary cells by GHRH expressed in pig primary myoblasts, we determined whether a single injection of 100 µg pSK-GHRH in adult immunocompetent C57B16 mice

would be sufficient to elicit enhanced GH systemic levels.

Five days before the plasmid administration, the mice were injected with 0.75% bupivacaine into the left quadriceps muscle. On day 0, animals were anesthetized and injected into the same muscle either with 100 µg of pSK-GHRH or pSK-LacZ in 100µl PBS. Three to 21 days later, the animals were weighted, killed, injected muscle collected and frozen in liquid nitrogen and the blood collected by transcardiac puncture.

The *in vivo* expression of pSK-GHRH was assessed by RT-PCR on injected muscle (**Figure 10**). Muscle RNA was DNase I treated in order to eliminate the injected plasmid, reextracted and 1 µg of total RNA was used in the reverse transcriptase reaction. Only the pSK-GHRH injected muscles showed a 254 bp PCR fragment when using GHRH specific primers. The pSK-LacZ injected muscles showed a 497 bp PCR fragment for mouse cytoskeletal  $\alpha$ -actin, used as a control, but not for GHRH. The efficiency of DNase treatment to eliminate plasmid DNA was checked using RNA from pSK-GHRH injected muscle. When the reverse transcriptase was omitted from the reaction, no amplification was seen.

Serum mGH in the pSK-GHRH quadriceps injected animals were significantly elevated compared with mGH levels in serum from control mice. Time course analysis of mGH as a response to pSK-GHRH injections showed stimulation at 3 days post-injection ( $21.54 \pm 15.29$  ng/ml vs.  $7.53 \pm 0.57$  ng/ml,  $n=6$ ), peaked at 7 days post-injection ( $36.28 \pm 27.28$  ng/ml vs.  $8.2 \pm 1.9$  ng/ml,  $p \leq 0.05$ ,  $n=6$ ) and declined gradually to the base-line by 21 days postinjection ( $9.16 \pm 2.54$  ng/ml vs.  $6.76 \pm 0.89$  ng/ml,  $n=6$ ).

Another indication of increased systemic levels of GH would be the linkage with the IGF-I biosynthesis in the liver. Thus, liver IGF-I expression of injected mice and controls was evaluated by Northern blot  
5 analysis of total RNA. Elevated mIGF-I mRNA expression was detected in all pSK-GHRH injected animals in comparison to a relatively stable baseline of IGF-I in RNA with those animals injected with pSK-LacZ. We observed increased IGF-I mRNA starting as soon as 3 days  
10 post-injection and maintained at least up to 21 days.

Finally, hGHRH secreted into the systemic circulation after intramuscular injection of pSK-GHRH enhanced growth in normal mice (**Figure 11**), as shown by significant differences in their total body mass at 14  
15 days ( $21.11 \pm 1.47\text{g}$  vs.  $18.62 \pm 0.4\text{g}$ ,  $p \leq 0.043$ ) and 21 days ( $21.86 \pm 1.45\text{g}$  vs.  $18.8 \pm 0.42\text{g}$ ,  $p \leq 0.028$ ) after a single injection of pSK-GHRH.

We observed only a transient activity of pSK-GHRH *in vivo*, after i.m. injection in adult mice, a fact most  
20 probably due to the humoral immune response targeted against hGHRH, a heterologous protein in mouse (Yao et al., 1994, *Gene Therapy* 1:99-107; Tripathy et al., 1996, *PNAS* 93:10876-10880; Tripathy et al, 1996, *Nature Med.* 2:545-550).

25 Our results demonstrate that the i.m. injected pSK-GHRH could be used to produce physiological levels of GHRH in the circulation of adult animals. This data suggests that it is possible to restore endogenous GH secretion to children and adults with GH deficiencies in  
30 a more physiological and less expensive way compared with the classical therapies.

#### Enhanced Vector Expression in Intact Muscle

Intact plasmid DNA in a sterile 20% sucrose solution (wt/vol) can be injected into mature avian or mammalian muscle. Following a single injection the vector DNA is stable for at least 30 days as a non-integrated extrachromosomal circular DNA in muscle nuclei and, is transcriptionally active. Wolf et al., *Science*, vol. 247, pp. 1465-1468 (1990). However, greater than 99% of the injected DNA is degraded in muscle under the Wolff protocol (Wolff, et al, *BioTechniques* 11:4374-485 (1991)). This protocol can be improved by increasing the uptake of plasmid DNA into muscle and reducing vector degradation. The procedure of the present invention can use expression vector DNA coated with the relevant transcriptional regulatory factors, the human serum response factor and other human associated nuclear proteins, such as histone, and transcription initiation factors to enhance uptake and stability. The regulatory proteins protect the DNA against muscle nucleases and facilitate the uptake of the protein coated DNA into myogenic nuclei.

The expression vector forms a protein/DNA complex by the sequence specific binding of the serum response factor with the inner core CCXXXXXXGG (where X can be either A or T; **SEQ ID NO. 3**) of the serum response element and by the addition of histone. The interaction with the inner core of the promoter facilitates myogenic cell type restricted expression of the skeletal  $\alpha$ -actin gene. The serum response factor, transcription initiation factor, transregulatory factor and histones are added to the expression vector by an *in vitro* binding reaction to form a reconstituted protein/DNA complex.

### Coating the Expression Vector System

A specific formulation involves coating the vector with elements of the transcription initiation complex and histone. This formulation is used both to enhance  
5 delivery of the vector to the cell and to enhance expression of the vector within the cell.

The following protocol was used to bacterially express and purify human serum response factor (SRF). Plasmid pARSRF-Nde is a T7 polymerase vector (Studier,  
10 F.W. and Moffatt, *J. Mol. Biol.* 189:113-130 (1986)) which produced full-length SRF protein upon IPTG (isopropyl-B-D-thiogalactopyranoside) induction. (Manak et al., *Genes and Development* 4:955-967 (1990)). E. coli BL21 harboring the plasmid was grown at 37°C to an  
15 OD<sub>600</sub> of 0.4 in TYP medium supplemented with ampicillin (50 µg/ml). Synthesis of SRF was then induced with 1mM IPTG for 2.0 hr, after which cells were spun down, washed once in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.0) and resuspended in a 40X packed cell volume and  
20 dialyzed against (10 mM HEPES [N-2 hydroxyethylpiperzine-N-2-ethansulfonic acid, pH 7.4], 60 mM KCl, 1mM 2-mercaptoethanol 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 10% glycerol). Cells were disrupted on ice by sonication. The lysate was  
25 clarified by centrifugation (15,000 xg for 20 min.) and the high speed supernatant containing overexpressed SRF was stored at -80C. Partial purification of SRF was done as follows. A 10 ml amount of the lysate was applied to a 10 ml phosphocellulose column equilibrated  
30 with column buffer (same as dialysis buffer as described above) and 0.05% Nonidet P-40. The flow through fractions were collected and applied to a 5-ml heparin agarose column. The column was washed with 0.35 M KCl

and SRF was eluted with 0.5 M KCl. SRF was then dialyzed and stored at -80°C.

Approximately, a ratio by weight of 5 to 1 SRF protein to expression vector DNA was allowed to incubate  
5 together in a solution containing 10 mM. Tris-HCl (pH 8.0, 0.1 mM EDTA, 2mM dithiothreitol, 5% glycerol plus 100 mM KCl. The binding of SRF to the actin promoter has been verified by DNA binding assays and by nuclease footprint protection assays as shown in the art.

10 Transcription initiation factors such as the TATA box protein (TBP) and other initiation factors such as TFIIB, E and F are eluted from purified HeLa cell nuclei by the protocol of Dignam et al., *Mol. Cell. Biol.* 10:582-598 (1983) with 0.42M KCl in the above dialysis  
15 buffer. Nuclear lysates containing transcription initiation factors are mixed together with the SRF-DNA plasmid at a ratio of 10 parts protein to one part SRF-DNA to help form a preinitiation complex which is dialyzed for 24 hours. Finally, a crude histone  
20 preparation which is stripped from HeLa nuclei in 6M urea, 2M NaCl is dialyzed against low salt dialysis buffer. The full complement of histone are slowly added to a final ratio of 1 to 1 (histone to the SRF-protein DNA complex) to form nucleosome particles over  
25 nonprotected DNA. The addition of histone will protect regions of DNA to a greater extent than naked DNA from cellular nucleases.

The nucleoprotein complex is then further formulated with a lipid base, nonaqueous base and/or  
30 liposomes for direct injection into muscle. Because of the abundance of specific transcription factors, which contain nuclear targeting sequences, expression vector DNA is readily delivered, and taken up into muscle

nuclei.

The vector can also be prepared in a formulation with other DNA binding compounds. For example, the vector can be prepared with polyvinyl pyrrolidone (PVP).

5 PVP is a synthetic polymer consisting of linear 1-vinyl-2-pyrrolidone groups. PVP is commercially available with various degrees of polymerization and molecular weights. Pharmaceutical grade PVP is marketed under the trade names Plasdone (International Specialty  
10 Products, ISP) and Kollidon (BASF). ISP describes the typical properties of Plasdone C-30 in its product literature. Plasdone C-30 has a weight average molecular weight of 50,000 g/mol.

PVP is found to interact with DNA by hydrogen  
15 bonding. PVP is also found to protect DNA *in vitro* from nuclease (DNase I) degradation. Reporter genes (CMV-CAT or CMV- $\beta$ -gal) were formulated in PVP solutions and injected into rat tibialis muscles after surgical exposure. The results showed that DNA formulated at 3  
20 mg/mL in 5% PVP in 150 mM NaCl led to the highest enhancement of gene expression over DNA formulated in saline. The levels of gene expression using lower molecular weight PVP (Plasdone C-15) were approximately 2-fold lower than levels of gene expression using  
25 formulations made with Plasdone C-30. When rat tibialis muscles were injected with DNA formulated in either saline or 5% PVP (Plasdone C-30), immunochemical staining for  $\beta$ -galactosidase revealed that the staining was more widely distributed in muscles treated with the  
30 formulated DNA. The staining also showed that the PVP formulation resulted in an increase in the number of cells expressing  $\beta$ -gal and that these cells were



distributed over a larger area as compared to DNA injected in saline. It is suggested that the increased tissue dispersion of DNA using PVP formulations is due to a hyper-osmotic effect in the muscle. DNA (3 mg/mL) in 5% PVP (Plasdone C-30) in 150 mM NaCl exerts an osmotic pressure of  $341 \pm 1$  mOsm/kg H<sub>2</sub>O.

An exemplary formulation of the hGHRH plasmid is a three-vial system, with product components to be mixed just prior to use. The product components are:

1. Human GHRH plasmid in sterile water;
2. Lyophilized PVP (polyvinylpyrrolidone; Plasdone C-30, Povidone U.S.P.); chemical formula  $(C_6H_9NO)_n$ ;
3. 115 mM sodium citrate buffer (pH 4) in 5% NaCl.

The expression vector can also be delivered as described below.

#### Administration

Administration as used herein refers to the route of introduction of a vector or carrier of DNA into the body. Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating disease by administration of the vector to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector and use of formulations for delivery are described above. The preferred embodiment is by direct injection using needle injection or hypospray.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the tissue specific DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months.

Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

Delivery can also be through use of DNA

transporters. DNA transporters refers to molecules which bind to DNA vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of noncovalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, e.g., the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo et al., U.S. Serial No. 07/855,389, entitled "A DNA Transporter System and Method of Use,, filed March 20, 1992, now abandoned; (2) Woo et al., PCT/US93/02725, International Publ. WO93/18759, entitled "A DNA Transporter System and method of Use", (designating the U.S. and other countries) filed March 19, 1993; (3) a continuation-in-part application by Woo et al., entitled "Nucleic Acid Transporter Systems and Methods of Use", filed December 14, 1993, U.S. Serial No. 08/167,641; (4) Szoka et al., U.S. Serial No. 07/913,669, entitled "Self-Assembling Polynucleotide Delivery System", filed July 14, 1992 and (5) Szoka et al., PCT/US93/03406, International Publ. WO93/19768 entitled "Self-Assembling Polynucleotide Delivery System", (designating the U.S. and other countries) filed April 5, 1993.

Transfer of genes directly into muscle has been very effective. Experiments show that administration by direct injection of DNA into muscle cells results in expression of the gene in the area of injection. Injection of plasmids containing GHRH results in expression of the gene for months at relatively constant levels. The injected DNA appears to persist in an unintegrated extrachromosomal state. This means of

transfer is the preferred embodiment.

Another preferred method of delivery involves a DNA transporter system. The DNA transporter system consists of particles containing several elements that are  
5 independently and non-covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations which may be used are spermine,  
10 spermine derivatives, histone, cationic peptides and/or polylysine. One element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein  
15 receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter  
20 system through a nuclear membrane. An example of such ligand is the nuclear targeting sequence from SV40 large T antigen or histone. A third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. Examples include inactivated  
25 virus particles such as adenovirus, peptides related to influenza virus hemagglutinin, or the GALA peptide described in the Skoka patent cited above.

Administration may also involve lipids. The lipids may form liposomes which are hollow spherical vesicles  
30 composed of lipids arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in

diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE which interact with DNA and with the membrane of the target  
5 cell to facilitate entry of DNA into the cell.

Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblasts  
10 genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

Myoblasts eventually differentiate and fuse to  
15 existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs gene therapy and the genetically engineered cells  
20 can also be easily put back without causing damage to the patient's muscle. Similarly, keratinocytes may be used to deliver genes to tissues. Large numbers of keratinocytes can be generated by cultivation of a small biopsy. The cultures can be prepared as stratified  
25 sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality over many years. The keratinocytes are genetically engineered while in culture by transfecting the keratinocytes with the appropriate vector. Although  
30 keratinocytes are separated from the circulation by the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

Delivery may also involve the use of viral vectors.

For example, an adenoviral vector may be constructed by replacing the E1 region of the virus genome with the vector elements described in this invention including  
5 promoter, 5'UTR, 3'UTR and nucleic acid cassette and introducing this recombinant genome into 293 cells which will package this gene into an infectious virus particle. Virus from this cell may then be used to infect tissue *ex vivo* or *in vivo* to introduce the vector  
10 into tissues leading to expression of the gene in the nucleic acid cassette.

The chosen method of delivery should result in expression of the gene product encoded within the nucleic acid cassette at levels which exert an  
15 appropriate biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be between 1-1000 mg/kg of body weight/day. This level is readily determinable by  
20 standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon disease delivery vehicle and efficacy data from  
25 clinical trials.

#### Cell Transfection and Transformation

One aspect of the present invention includes cells transfected with the vectors described above. Once the  
30 cells are transfected, the transformed cells will express the protein or RNA encoded for by the nucleic acid cassette. Examples of proteins include, but are not limited to polypeptide, glycoprotein, lipoprotein,

phosphoprotein, or nucleoprotein.

The nucleic acid cassette which contains the genetic material of interest is positionally and sequentially oriented within the vectors such that the  
5 nucleic acid in the cassette can be transcribed into RNA and, when necessary, be translated into proteins or polypeptides in the transformed cells.

A variety of proteins can be expressed by the sequence in the nucleic acid cassette in the transformed  
10 cells. Those proteins which can be expressed may be located in the cytoplasm, nucleus, membranes (including the plasmalemma, nuclear membrane, endoplasmic reticulum or other internal membrane compartments), in organelles (including the mitochondria, peroxisome, lysosome,  
15 endosome or other organelles), or secreted. Those proteins may function as intracellular or extracellular structural elements, ligand, hormones, neurotransmitter, growth regulating factors, differentiation factors, gene-expression regulating factors, DNA-associated  
20 proteins, enzymes, serum proteins, receptors, carriers for small molecular weight organic or inorganic compounds, drugs, immunomodulators, oncogenes, tumor suppressor, toxins, tumor antigens, or antigens. These proteins may have a natural sequence or a mutated  
25 sequence to enhance, inhibit, regulate, or eliminate their biological activity. A specific example of a protein to be expressed is hGHRH.

In addition, the nucleic acid cassette can code for RNA. The RNA may function as a template for  
30 translation, as an antisense inhibitor of gene expression, as a triple-strand forming inhibitor of gene expression, as an enzyme (ribozyme) or as a ligand recognizing specific structural determinants on cellular

structures for the purpose of modifying their activity.

Specific examples include RNA molecules to inhibit the expression or function of prostaglandin synthase, lipo-oxenganse, histocompatibilty antigens (class I or class  
5 II), cell adhesion molecules, nitrous oxide synthase,  $\beta_2$  microglobulin, oncogenes, and growth factors.

The compounds which can be incorporated are only limited by the availability of the nucleic acid sequence for the protein or polypeptide to be incorporated. One  
10 skilled in the art will readily recognize that as more proteins and polypeptides become identified they can be integrated into the vector system of the present invention and expressed in animal or human tissue.

Transfection can be done either by *in vivo* or *ex vivo* techniques. For example, muscle cells can be  
15 propagated in culture, transfected with the transforming gene, and then transplanted into muscle tissue. Alternatively, the vectors can be administered to the cells by the methods discussed above.

20

#### Methods of Use

##### A. Treatment with Growth Hormone Releasing Hormone

Growth hormone is normally produced and secreted  
25 from the anterior pituitary and promotes linear growth in prepuberty children. Growth hormone acts on the liver and other tissues to stimulate the production of growth hormone releasing hormone. This factor is, in turn, responsible for the growth promoting effects of  
30 growth hormone. Further, this factor serves as an indicator of overall growth hormone secretion. Serum IGF-I concentration increases in response to endogenous and exogenous administered growth hormone. These



concentrations are low in growth hormone deficiency.

Growth hormone releasing hormone is one of the key factors that potentiates muscle development and muscle growth. Myoblasts naturally secrete GHRH as well as its cognate binding proteins during the onset of fusion. This process coincides with the appearance of muscle specific gene products. In terminally differentiated muscle, signals propagated from passive stretch induced hypertrophy induce the expression of IGF genes. Many of the actions of IGFs on muscle result from interactions with the GHRH receptor.

The intramuscular injection of an expression vector containing the sequence for GHRH (for example, pSK-GHRH) can be used to treat growth disorders. Vectors are designed to preferably control the expression of GHRH in a range of 0.1-10 ng/ml. Since intramuscular expression of vectors leads to expression of the product encoded by the nucleic acid cassette for several months, this method provides a long-term inexpensive way to increase systemic blood concentration of GHRH and consequently GH and IGF-I in patients with growth hormone deficiency.

### B. Treatment of Muscle Atrophy Due To Age

Growth hormone levels decline with increasing age.

The levels in healthy men and women above age of 55 are approximately one third lower than the levels in men and women 18 to 33. This is associated with a decrease in the concentration of IGF-I. The decline in growth hormone and IGF-I production correlate with the decrease in muscle mass, termed senile muscle atrophy, and increase in adiposity that occur in healthy human subjects. Administering growth hormone three times a week to healthy 61 to 81 year old men who had serum levels below those of healthy younger men increased the serum IGF-I levels to within the range found in young healthy adults. This increased level led to increased muscle mass and strength and reduced body fat. The secretion of growth hormone is regulated by a stimulatory (growth hormone releasing hormone) and an inhibitory (somatostatin) hypothalamic hormone.

The convenient cloning sites in the expression vectors of the present invention are used to construct vectors containing human growth hormone CDNA sequence, the human growth hormone releasing hormone (GHRH), or IGF-I. This versatility is important since the GHRH, GH, and IGF-I, while having similar desired effects on muscle mass, may have different side effects or kinetics which will affect their efficacy. The expression of the growth hormone releasing hormone might be more advantageous than the expression of either IGF-I or the growth hormone vectors transcripts. Since GHRH is reduced in the elderly it appears to be responsible for the lack of GH secretion rather than the anterior pituitary capability of synthesizing growth hormone, thus the increased expression of GHRH from muscle would

increase GHRH levels in the systemic blood system and can allow for the natural diurnal secretion pattern of GH from the anterior pituitary. In this way, GHRH could act as the natural secretagogue, allowing for elevated secretion or release of GH from the hypothalamus of the elderly.

Thus, the application of vector systems described herein to express growth hormone releasing hormone through the injection of the pSK-GHRH or related vectors, vectors expressing HG, or IGF-I into adult muscle of the elderly is a long-term inexpensive way to increase systemic blood concentration of IGF-I in the elderly.

Administration of the vectors can be intravenously, through direct injection into the muscle or by any one of the methods described above. Dosages will depend on the severity of the disease and the amount of dosage is readily determinable by standard methods. The duration of treatment will extend through the course of the disease symptoms which can be continuously.

#### C. Treatment of Osteoporosis

Osteoporosis is a common accelerated loss of bone mass that often accompanies aging. The decreased bone density associated with osteoporosis leads to an increased susceptibility to bone fractures. Treatment with IGF-I is associated with increased bone density. Thus, administration of a vector encoding GHRH to muscles by direct injection or hypospray will induce a higher level of IGF-I production and will thereby aid in the redeposition of bone and thereby decrease the risk of fractures.

Administration of the vectors can be intravenously,

through direct injection or by any one of the methods described above. Dosages will depend on the severity of the disease and the amount of dosage is readily determinable by standard methods. The duration of treatment will extend through the course of the disease symptoms which can be continuously.

#### D. Treatment of Cachexia

Muscle wasting (cachexia, negative nitrogen balance, loss of lean body mass) is a common complication of a number of chronic diseases, such as AIDS, cancer, and rheumatic disease. This process contributes substantially to a morbid cycle of inactivity, malnutrition, and opportunistic infections, resulting in prolonged disability, extended hospitalization, and considerable health care expense. Muscle wasting is also a common feature of morbid ageing and it is likely that measures to preserve muscle mass would have a substantial beneficial impact in the morbidly ageing population. Reversal of muscle wasting may be an efficient method for treating osteoporosis as well.

Current therapies focus on dietary management with the use of high calorie dietary supplements or parenteral nutrition or use of appetite stimulants. Dietary approaches are inherently limited by the poor utilization of caloric intake in these patients. Androgens are theoretically effective but have profound side effects which complicate their use. Thus, there is a need for an effective medicinal approach which directly promotes preservation of muscle mass. Preferably the approach will involve a therapeutic composition which does not require frequent

administration, thus providing improved compliance in chronically ill and ageing populations.

IGF-1 is the major growth factor promoting the differentiation of muscle cells and increasing muscle mass. Studies in animals demonstrate that IGF-1 will effectively reverse cachexia, though this requires chronic (ideally continuous) administration. hGH has also been shown to preserve lean body mass in animal studies and is in use in many clinical trials for this indication.

The stimulation of GH and IGF-1 for the treatment of cachexia can advantageously be provided by the *in vivo* expression of GHRH from a vector, e.g., the pSK-GHRH vector, thereby avoiding the difficulties associated with direct administration of IGF-1 or GH. This method is expected to be particularly advantageous in cases of systemic muscle wasting. The vector can be administered by various methods, such as those indicated above. An example of such an administration method is the direct injection of a composition containing the vector encoding GHRH in muscle tissue of the patient to be treated.

#### Improvement of Livestock

An additional embodiment of the present invention is the improvement of livestock by injection of GHRH vector constructs, or similar constructs encoding other growth hormones, such as growth hormone or growth hormone releasing hormone. It has been shown that GHRH stimulates milk production (galactopoietic) with no alteration in milk composition, and sustains growth, mostly on the behalf of lean body mass, in farm animals (Enright et al., 1993, *J. Animal Science* 71:2395-2405;

Enright et al., 1986, *J. Dairy Science* 69:344-351). Thus, muscle injection of vectors encoding GHRH by hypodermic or hypospray administration will promote increased muscle mass and reduced body fat in important livestock species such as cattle, sheep, swine, rabbits, deer, fish and birds such as turkeys, chickens, ducks, and geese. Likewise, milk production can also be stimulated by *in vivo* expresssion of GHRH from vectors such as those described above. Administration of the vectors can also be through any one of the methods described above.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

#### Example 1

##### Construction of pSK-GHRH and pSK-LacZ

The plasmid DNA backbone was pBlueScript KS+ (pBS). In order to construct hybrid pSK-GHRH, a 448bp fragment (-424/+24) of avian skeletal  $\alpha$ -actin promoter (SK) (Lee, T.C. et al., *Oncogene* 9:1047-1052 (1994); Chow, K.L. et al., *Proc. Natl. Acad. Sci. USA*, 88:1301-1305 (1991)) was cloned upstream from the hGHRH cDNA 228 bp fragment (part of exon 2, all exon 3 and part of exon 4) coding for the 31 amino acid signal peptide and entire mature hGHRH1-44 peptide (Tyr1-Leu44) inserted into the BamHI / HindIII sites of pBS derived plasmid. The 3' untranslated region of hGH cDNA in a 622bp SmaI / EcoRI blunted fragment, was excised from the commercial pOGH plasmid (Nichols Institute) and cloned into blunted-ended ClaI / EcoRI sites of pBS derived plasmid. In the pSK-LacZ construct, the  $\beta$ -galactosidase gene of

Escherichia coli, with a nuclear localization signal (nls), is driven by the same SK promoter, but contains the 3' UTR of skeletal  $\alpha$ -actin gene.

5

### Example 2

#### In Vitro Expression of pSK-GHRH

A plasmid vector which is capable of directing high-level gene expression in a skeletal muscle specific manner is generated as follows. A 228 bp fragment of  
10 hGHRH, which encode for the 31 amino acid signal peptide and the entire mature peptide hGHRH(1-44)OH (Tyr1→Leu44), was cloned into a pBS-derived vector. Gene expression was controlled by a 448bp fragment (-424 / +24) of the avian skeletal  $\alpha$ -actin gene, which  
15 contains several evolutionarily conserved regulatory elements that accurately initiate skeletal  $\alpha$ -actin transcripts and drives transcription of a variety of reporter genes specifically in differentiated skeletal muscle cells. The GHRH coding region was followed by  
20 the 3' untranslated region of human growth hormone cDNA.

### Example 3

#### In Vitro Expression of pSK-GHRH

In vitro expression of pSK-GHRH was examined in  
25 transiently transfected chicken primary myoblasts. pSK-GHRH and pSK-LacZ transfected cells were placed into differentiation media for 24-72h to initiate withdrawal from the cell cycle and to induce post-fusion differentiation. The media was changed to a minimal  
30 serum-free media for a 24h pulse. Cells were harvested 48 to 96h post-differentiation. Northern blot analysis (Figure 12) showed the expected size transcripts of

0.35kb, in myoblasts transfected with pSK-GHRH, but not in pSK-LacZ transfected myoblasts. The expression of pSK-GHRH peaked at 48h post-differentiation and was reduced thereafter in comparison to the glycolytic enzyme GAPDH. This pattern of activation is characteristic for chicken skeletal  $\alpha$ -actin promoter, which induces high levels of transgene expression in myotubes but not in replicating myoblasts.

Conditioned serum-free media from pSK-GHRH and pSK-LacZ transfected myoblasts were collected and purified on C18 Sep-Columns, which served to separate the peptide to be assayed from potentially interfering substances and to concentrate the samples to determine levels of radioimmunoassayable hGHRH. Chicken primary myoblasts transfected with pSK-GHRH produced approximately 1.7 ng hGHRH(1-44)OH / million cells / h.

#### Example 4

##### In Vitro Activity of pSK-GHRH

The *in vitro* potency of the hGHRH (1-44)OH molecule secreted by primary pig myoblasts (ppm) transiently transfected with pSK-GHRH was compared to that of the hGHRH (1-44)NH<sub>2</sub> synthetic molecule for its ability to stimulate GH release from primary pig anterior pituitary cells after a 24h stimulation at 37°C. GH release from the primary pig anterior pituitary cells rose from values of  $7 \pm 2$  ng/ml to  $82.5 \pm 3.1$  ng/ml ( $p \leq 0.002$ ) when stimulated with 5 ml culture media from 1 million primary pig myoblasts transiently transfected with pSK-GHRH, containing a radioimmunoassay equivalent estimated to be 10ng of hGHRH (1-44)OH. This value equals 72% of that obtained when the pituitary cells were stimulated



with myoblast serum-free media mixed with 10ng synthetic hGHRH (1-44)NH<sub>2</sub> then purified and processed as the test media (GH release in this case was 115 ± 3.2 ng/ml). Thus, hGHRH(1-44)OH secreted by skeletal pig myocytes  
5 transfected with pSK-GHRH retain functional activity in pig pituitary cell culture and induces secretion of significant levels of GH. This is an important finding, and contracts with prior art technique. Pig (1-44)GHRH, as well as the human molecule, is amidated, while the  
10 molecule which is expressed by the present construct is non-amidated at amino acid 44.

#### Example 5

##### In Vivo Expression and Activity of pSK-GHRH

15 On day 0, animals were anesthetized and injected with 100 µ of pSK-GHRH of pSK-LacZ in 100 µ PBS into the regenerating quadriceps muscle. The animals were killed over the next 3 weeks and samples of the injected muscles were collected and frozen in liquid nitrogen and  
20 blood collected by transcardiac puncture.

The *in vivo* expression of pSK-GHRH was assessed by RT-PCR on injected muscle (**Figure 13**). Muscle RNA was DNase I treated in order to eliminate the injected plasmid, reextracted and 1 mg of total RNA was used in  
25 the reverse transcriptase reaction. Only the pSK-GHRH injected muscles showed a 254 bp PCR fragment when amplified with GHRH specific primers ([+]pSK-GHRH). The pSK-LacZ injected muscles showed a 497 bp PCR fragment for mouse cytoskeletal β-actin, used as a control, but  
30 not for GHRH. The efficiency of DNase treatment to eliminate plasmid DNA was screened by using RNA from pSK-GHRH injected muscle: when the reverse

transcriptase was omitted from the reaction, no amplification was observed((-) pSK-GHRH).

Serum mGH in the pSK-GHRH injected animals was significantly elevated as compared to the mGH levels in serum from control mice (**Figure 14**). Time course analysis of mGH as a response to pSK-GHRH injections showed stimulation at 3 days post-injection ( $21.54 \pm 15.29$  ng/ml vs.  $7.53 \pm 0.57$  ng/ml,  $n=6$ ), peaked at 7 days post-injection ( $36.28 \pm 27.28$  ng/ml vs.  $8.2 \pm 1.9$  ng/ml,  $p \leq 0.05$ ,  $n=6$ ) and declined gradually to the base-line by 21 days post-injection ( $9.16 \pm 2.54$  ng/ml vs.  $6.76 \pm 0.89$  ng/ml,  $n=6$ ).

Another indication of increased systemic levels of GH would be elevated IGF-1 biosynthesis in the liver. Thus, liver IGF-1 expression of injected mice and controls was evaluated by Northern blot analysis of total RNA (**Figure 15**). Elevated mIGF-1 mRNA expression was detected in all pSK-GHRH injected animals in comparison to a relatively stable baseline of IGF-1 RNA in pSK-LacZ injected mice. IGF-1 mRNA levels increased within 3 days post-injection and was maintained up to 21 days.

hGHRH secreted into the systemic circulation after intramuscular injection of pSK-GHRH enhanced growth in normal mice (**Figure 16**), as shown by significant differences in their total body mass at 14 days ( $21.11 \pm 1.47$ g vs.  $18.62 \pm 0.4$ g,  $p \leq 0.043$ ) and 21 days ( $21.86 \pm 1.45$ g vs.  $18.8 \pm 0.42$ g,  $p \leq 0.028$ ) after a single injection of pSK-GHRH. These results demonstrate that the i.m. injection of pSK-GHRH can be used to produce physiological levels of GHRH in the circulation of adult animals.

However, the hGHRH is unsuitable for use in other animals. Only a transient increase was observed in mGH *in vivo*, after i.m. injection of pSK-GHRH in adult mice.

This response is due to a humoral immune response  
5 targeted against hGHRH, which is a heterologous protein in mouse. The antibody response to hGHRH was demonstrated using an ELISA assay (**Figure 17**). Antibodies to hGHRH were detected in sera collected 21 days and 28 days after intramuscular injection of pSK-GHRH but not in sera collected 21 days after  
10 intramuscular injection of pSK-LacZ ( $p \pm 0.05$ ). Also, the persistence of hGHRH transcripts in the muscle 21 days after injection demonstrate that expression persists and that the decline of serum hGHRH concentrations is due to  
15 the humoral immune response.

Cloning the cDNA coding for the hGHRH(1-40)OH molecule, which is naturally hydroxylated and has the same potency *in vivo* as hGHRH(1-44)NH<sub>2</sub> provides greater GH release in plasmid injected animals. In addition,  
20 mutation of some of the amino acids which are known to be sites for different peptidases, prolong the half-life of the hGHRH molecule. In order to regulate the expression level *in vivo* and to obtain, if necessary, a discontinuous release of GHRH, a gene switch (Wang, Y.  
25 *et al.*, *Nature Biotechnology* 15:239-243 (1997)) is an important element to be added.

The lost cost, the possibility of large scale production of plasmid DNA, combined with the easy administration procedure and the 10-20 times higher  
30 potency at the same dose as compared to GH, provides utility for agricultural uses. The intramuscular plasmid delivery represents a practical way to improve performance of domestic animals and provide an

alternative to classical GH treatments.

A GHRH plasmid delivery, which avoids the frequent administration of recombinant proteins currently used in agriculture and human clinics and provides a more  
5 natural alternative for the GH-based therapies.

### Example 6

#### Cell Culture

Minimal Essential Medium (MEM), heat inactivated  
10 horse serum (HIHS), gentamicin, Hank's Balanced Salt Solution (HBSS), lipofectamine were obtained from Gibco BRL. The skilled artisan recognizes that primary chicken myoblast cultures can be obtained (Bergsma, D.J. *et al.*, *Molecular & Cellular Biology* 6:2462-2475  
15 (1986)). Cells were plated 24h prior to transfection at a density of 1.5 million cells / 100mm plate, in MEM supplemented with 10% HIHS, 5% chicken embryo extract (CEE) and gentamicin. Cells were maintained in a humidified 5% CO<sub>2</sub> 95% air atmosphere at 37°C. Cells were  
20 transfected with 4  $\mu$  of pSK-GHRH or pSK-LacZ per plate using lipofectamine, according to the manufacturer instructions. After transfection, the medium was changed to MEM which contained 2% HIHS, 2% CEE for at least 24h to allow the cells to differentiate. Primary  
25 pig myoblast cultures were obtained as described in Doumit, M.E. *et al.*, *Tissue & Cell* 24:253-262 (1992). Cells were plated at a density of 1 million cells / 100mm plate and maintained in growth media for 2-3 days.  
The cells were passed for 2 times prior to  
30 transfection. Porcine myoblast cultures were transfected and differentiated under the same conditions as the primary chicken myoblast cultures. Media and

cells were harvested 48, 72 and 96h postdifferentiation. The samples and controls were assayed in quadruplicate. The efficiency of transfection was estimated by  $\beta$ -galactosidase histochemistry of control plates to be 10%. One day before harvesting, cells were washed twice in HBSS and the media changed to MEM, 0.1% BSA. The cells were homogenized directly into Ultraspec RNA reagent (Biotech Laboratories) for the isolation of total RNA. Conditioned media was treated by adding 0.25 volume of 1% trifluoroacetic acid (TFA) and 1mM phenylmethylsulfonylfluoride (PMSF), frozen at  $-80^{\circ}$ , lyophilized, purified on C-18 Sep-Columns, lyophilized and used in RIA or resuspended in media conditioned for primary pig anterior pituitary culture. The pit anterior pituitary culture was obtained as described (Tanner, J.W. et al., *J. Endocrinol* 125:109-115 (1990)). Pig GH was assayed as described in Barb, C.R. et al., *Domestic Animal Endocrinology* 8:117-127 (1991).

20

### Example 7

#### Northern Blot Analysis

10-20  $\mu$  of total RNA was DNase I treated (Gibco BRL), size separated in 1.5% agarose-formaldehyde gel and transferred to Gene Screen nylon membrane (DuPont Research Products). The membranes were hybridized with cDNA probes  $^{32}$ P labeled by random priming (Ready-to-Go DNA labeling kit, Pharmacia Biotech). Hybridization was carried out at  $45^{\circ}\text{C}$  in a solution which contained 50% formamide, 5xSSPE, 5xDenhardt's, 1% SDS, 200 mg/ml sheared salmon sperm DNA. Membranes were washed twice for 10 minutes in 2xSSPE/1%SDS at room temperature and twice for 30 minutes in 0.2xSSPE/1%SDS at  $68^{\circ}\text{C}$ . Blots

were subsequently exposed to X-ray film (Kodak X-Omat AR) at -80°C with intensifying screens.

#### Example 8

#### 5           Intramuscular Injection of Plasmid DNA in Adult                   Mice

C57/B16 male mice (Taconic Laboratories) were housed under environmental conditions of 10h light / 14h darkness. On day -5, the left quadriceps muscle of mice  
10 (17-20g body weight) was injected with 50 µ of 0.75% bupivacaine hydrochloride in saline solution. On day 0 the animals were weighed, the regenerating muscle was exposed and injected with 100 µ PBS. The animals were weighed and killed 3-21 days later. Blood was  
15 collected, centrifuged immediately at 0°C, and stored at -80°C prior to analysis. Injected and control organs were removed and frozen in liquid nitrogen.

#### Example 9

#### 20           RT-PCR

Muscle RNA was extracted with Ultraspec RNA reagent. 1 µ of total RNA was treated twice with 10 units of DNase I (Gibco BRL) and phenol-chloroform extracted. RNA pellets were resuspended in 20 µ DEPC-  
25 water. Reverse transcriptase reactions were performed with the SuperScript Preamplification System for First Strand cDNA Synthesis (Gibco BRL) according to manufacturer instructions. In (-) pSK-GHRH tubes the reverse transcriptase was omitted. Specific  
30 oligonucleotide primers were used to amplify either a 254 bp fragment of pSK-GHRH cDNA:  
5'TGGTGCTCTGGGTGTTCTT3' (sense) (SEQ ID NO.12) and

5 'GCTTGATATCGAATTCCTGC3' (anti-sense) (SEQ ID NO.13) or  
a 497bp control fragment of mouse cytoskeletal  $\beta$ -actin  
cDNA: 5'TCAGAAGGACTCCTATGTGG3' (sense) (SEQ ID NO.14)  
and 5'TCTCTTTGATGTCACGCACG3' (anti-sense) (SEQ ID  
5 NO.15). The PCR conditions were, 32 cycles (94°C for  
30s, 60°C for 30s, 72°C for 1min) in 50  $\mu$  containing 5  $\mu$   
of final volume of RT reaction diluted in 1x PCR buffer  
which contained 1.5 mM MgCl<sub>2</sub>, 200 mM each dNTP, 2.5 units  
AmpliTaq DNA polymerase (Perkin-Elmer) and 100 ng of  
10 each specific primer.

#### Example 10

##### Mouse Growth Hormone RIA

Mouse GH in plasma was measured with a heterologous  
15 rat assay system (Amersham). The sensitivity of the  
assay was 0.16 ng/tube. The intra- and interassay  
coefficients of variation were 6.5 and 6.8%  
respectively.

#### Example 11

##### Detection of Mouse anti-hGHRH Antibodies

20 Mouse anti-hGHRH antibodies were detected by ELISA.  
Ninety-six-well plates (Dynatech Laboratories) were  
coated with 500 ng of purified hGHRH (Peninsula  
25 Laboratories) per well (in HEPES buffered saline) at 4°C  
overnight. The wells were washed five times with PBS,  
blocked with PBS containing 5% (w/v) non fat dry milk  
and then incubated 2 hours at room temperature with  
serial dilutions of serum (in PBS + 2%BSA) from pSK-GHRH  
30 or pSK-LacZ injected mice. The wells were washed five  
times with PBS and then incubated with 50 ml of a 1:2000  
dilution of HRP-conjugated goat anti-mouse IgG for 2

hours at room temperatures. 200  $\mu$  of peroxidase developing reagent (ABTS substrate) were incubated for 1 hour at room temperature. Plates were read at 410 nm in a Dynatech MR600 plate reader. In this assay, a rabbit  
 5 anti-hGHRH antibody used with a HRP-conjugated goat anti-rabbit secondary antibody was the positive control for sensitivity.

### Example 12

10

### Statistics

Data were analyzed using Microsoft Excel statistics analysis package. Specific p values were obtained by comparison using Student's t test. A value of  $p \leq 0.05$  was taken to be statistically significant. Values shown  
 15 in the figures are the mean  $\pm$  s.e.m.

**Table 1**

**Mouse growth hormone values in pSK-GHRH injected mice and controls (ng/ml)**

	GH	LacZ	AvGHRH	AvLacZ
day 3	6.8	7.2		
	9	8.2		
	12	6.2		
	38	8.2	16.45	7.45
day 5	11	7.8		
	24	10	17.5	8.9
day 7	12	7.2		
	70	10		
	28	12		
	60	7.8	42.5	9.25
day 10	17	12.4		
	18	8	17.5	10.2
day 14	17	7.5		



	5.6	7.4		
	17.6	12		
	7.4	8.3	11.9	8.8
day 21	9	6.2		
	6.2	6.3		
	13	7.8	9.4	6.766667

**Table II****Identification of GHRH Sequences**

<b>SEQ ID NO.5</b>	Bovine growth hormone releasing hormone sequence
<b>SEQ ID NO.6</b>	Porcine growth hormone releasing hormone sequence
<b>SEQ ID NO.7</b>	Ovine growth hormone releasing hormone sequence
<b>SEQ ID NO.8</b>	Mouse growth hormone releasing hormone sequence
<b>SEQ ID NO.9</b>	Caprine growth hormone releasing hormone sequence
<b>SEQ ID NO.10</b>	Human 1-40 OH growth hormone releasing hormone sequence
<b>SEQ ID NO.11</b>	Mouse/porcine chimeric growth hormone releasing hormone sequence

5           One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The vector systems along with the methods, procedures treatments and

10   vaccinations described herein are presently representative of preferred embodiments are exemplary and not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within

15   the spirit of the invention or defined by this scope with the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be

made to the invention disclosed herein within departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All  
5 patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

Claims

We claim:

1. A vector for expression of a nucleic acid sequence in a cell, comprising:
  - 5 a nucleic acid cassette containing a nucleotide sequence encoding GHRH;  
a 5' flanking region including one or more sequences necessary for expression of said nucleic acid cassette, wherein said sequences include a promoter;
  - 10 a linker connecting said 5' flanking region to a nucleic acid, said linker having a position for inserting said nucleic acid cassette, wherein said linker lacks the coding sequence of a gene with which it is naturally associated; and
  - 15 a 3' flanking region, including a 3'UTR or a 3'NCR or both, wherein said 3' flanking region is 3' to said position for inserting said nucleic acid cassette, and wherein said 3' flanking region comprises a sequence from a 3'-UTR.
- 20 2. The vector of claim 1, wherein said GHRH is human GHRH.
3. The vector of claim 2, wherein said nucleotide  
25 sequence encoding for human GHRH is a synthetic sequence.
4. The vector of claim 3, wherein said nucleotide sequence encoding for human GHRH has the sequence of  
30 **SEQ ID NO. 2.**
5. The vector of claim 1, wherein said promoter is a promoter from a skeletal  $\alpha$ -actin gene.

6. The vector of claim 5, wherein said promoter from a skeletal  $\alpha$ -actin gene is from a chicken.

5 7. The vector of claim 5, wherein said promoter from a skeletal  $\alpha$ -actin gene is from a human.

8. The vector of claim 1, wherein said 3'-UTR is a growth hormone 3'-UTR.

10

9. The vector of claim 8, wherein said growth hormone 3'-UTR is from a human growth hormone gene.

10. The vector of claim 8, wherein an ALU repeat  
15 or ALU repeat-like sequence is deleted from said 3' UTR.

11. The vector of claim 1, wherein said GHRH is human GHRH, said promoter is from a chicken skeletal  $\alpha$ -actin gene, and said 3'-UTR is from a human growth  
20 hormone gene.

12. The vector of claim 1, wherein said 5' flanking region or said 3' flanking region or both regulates expression of said nucleic acid cassette  
25 predominately in a specific tissue.

13. The vector of claim 12, wherein said specific tissue is myogenic.

30 14. The vector of claim 1, wherein said 5' flanking region includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in

appropriate relationship for expression of said nucleic acid cassette.

15        15. The vector of claim 14, wherein said 5' flanking region further comprises a 5' mRNA leader sequence inserted between said promoter and said nucleic acid cassette.

10        16. The vector of claim 1, wherein said vector further comprises an intron/5' UTR from a chicken skeletal  $\alpha$ -actin gene.

15        17. The vector of claim 1, wherein said vector further comprises an antibiotic resistance gene.

18. The vector of claim 1, wherein said vector comprises a nucleotide sequence which is the same as the nucleotide sequence of plasmid pSK-GHRH.

20        19. A formulation for delivery and expression of a human GHRH gene in a cell, said formulation comprising a vector of claim 1 in a solution having between 0.5% and 50% PVP.

25        20. The formulation of claim 19, wherein said solution includes about 5% PVP.

30        21. A transgenic animal having a plurality of cells containing the vector of claim 1.

22. The transgenic animal of claim 21, wherein said cell is a germ or somatic cell.

23. A cell transformed with a vector of claim 1.

24. The transformed cell of claim 23, wherein said cell is myogenic.

5

25. A method for transfection of a cell *in situ*, comprising the step of contacting said cell with a vector of claim 1 for sufficient time to transfect said cell.

10

26. The method of claim 25, wherein transfection of said cell is performed *in vivo*.

27. The method of claim 26, wherein said  
15 contacting is performed in the presence of an about 5% PVP solution.

28. The method of claim 25, wherein transfection of said cell is performed *ex vivo*, further comprising  
20 the steps of cotransfecting said vector with a selectable marker and selecting the transformed cells.

29. A method for delivery and expression of a GHRH gene in a plurality of cells, comprising the steps of:

25 (a) transfecting said plurality of cells with a vector of claim 1; and

(b) incubating said plurality of cells under conditions allowing expression of a nucleic acid sequence in said vector, wherein said nucleic acid  
30 sequence encodes GHRH.

30. The method of claim 29, wherein said GHRH is hGHRH and said cells are human cells.

31. The method of claim 30, wherein said contacting is performed in the presence of an about 5% PVP solution.

5

32. A method for treating a disease or condition, comprising the steps of transfecting a cell *in situ* with a vector of claim 1.

10 33. The method of claim 32, wherein said disease or condition is a localized disease or condition.

34. The method of claim 32, wherein said disease or condition is a systemic disease or condition.

15

35. The method of claim 32, wherein said disease or condition to be treated is selected from the group consisting of osteoporosis, cachexia, and growth disorders.

20

36. A method of expressing growth hormone releasing (GHRH) in a non-human vertebrate animal comprising the step of: inserting a DNA carrier vehicle containing a gene sequence encoding a growth hormone releasing hormone polypeptide sequence operatively linked to a vertebrate gene promoter into said non-human vertebrate animal tissue under conditions where said gene is expressed and produces growth hormone releasing hormone.

25 30 37. The method of claim 36 wherein said gene

sequence encodes for a growth hormone releasing hormone having one of the following sequences;

SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, or SEQ ID NO.11.

5

38. The method of claim 36 wherein said vertebrate animal is one of the following species; porcine, bovine, equine, canine, feline, caprine, avian (chicken, turkey, duck), ovine or fish.

10

39. The method of claim 36 wherein the gene sequence in said DNA carrier vehicle contains no intervening sequences.

15 40. The method of claim 36, wherein said promoter is from a skeletal  $\alpha$ -actin gene.

41. The method of claim 36, wherein said DNA carrier vehicle is injected into said animal muscle.

20

42. The method of claim 36, wherein said DNA carrier vehicle is a plasmid DNA vector capable of infecting said vertebrate animals in various tissues.

25 43. The method of claim 36, wherein said DNA carrier vehicle is an adenovirus or adeno-associated virus capable



of infecting said vertebrate animals in various tissues.

44. The method of claim 43, wherein the promoter -  
GHRHcDNA-3'UTR is incorporated into said adeno-associated  
5 virus.

45. The method of claim 36, wherein said vectors  
encode for an Arg-Arg sequence before a tyrosine or a  
histidine.  
10

46. The method of claim 36, wherein said DNA carrier  
vehicle includes a gene switch.

47. The method of claim 36, wherein said gene  
15 sequence is a chimeric synthetic cDNA encoding GHRH  
comprising a mouse specific fragment and a species  
specific fragment and wherein the mouse specific fragment  
contains the first 45 nucleotides and encodes the first 15  
amino acids of the mouse GHRH, and said mouse specific  
20 fragment is fused in frame with the species-specific  
fragment contains 87 nucleotides and encodes the 16th to  
44th amino acids of a species-specific GHRH, said chimeric  
sequence providing resistance against dipeptidases.

25 48. The method of claim 47, wherein said species-  
specific fragment for GHRH encodes a polypeptide from one

of the following animal species; porcine, bovine, equine, canine, feline, caprine, avian (chicken, turkey, duck) ovine or fish.

- 5           49. The method of claim 47, wherein said species-specific fragment of GHRH encodes DNA sequence encodes for one of the following GHRH polypeptides; SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, or SEQ ID NO.11.

RESTRICTION MAP OF THE CHICKEN  
SKELETAL ALPHA ACTIN GENE

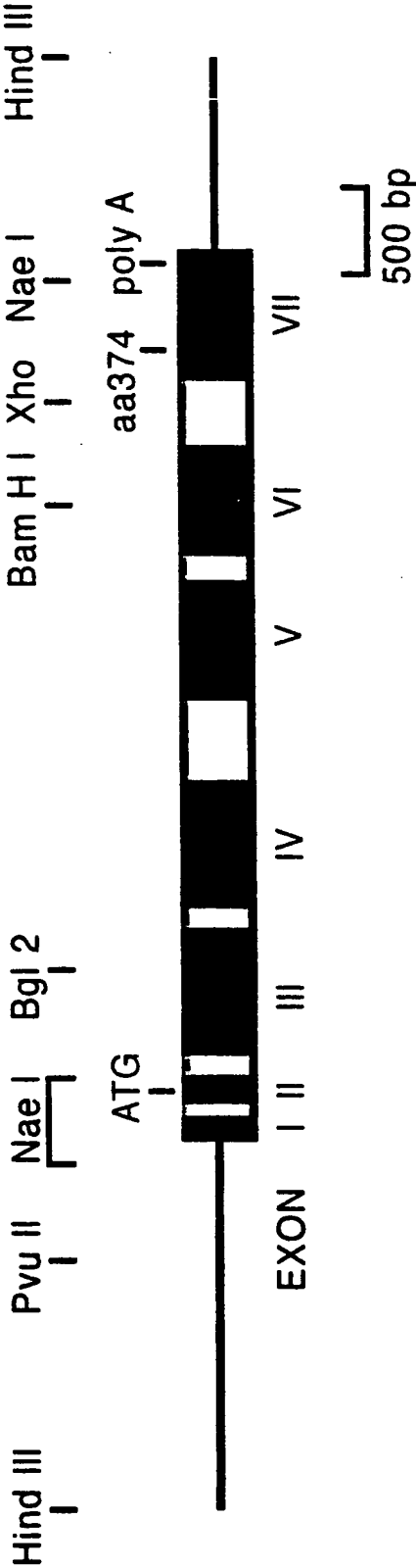
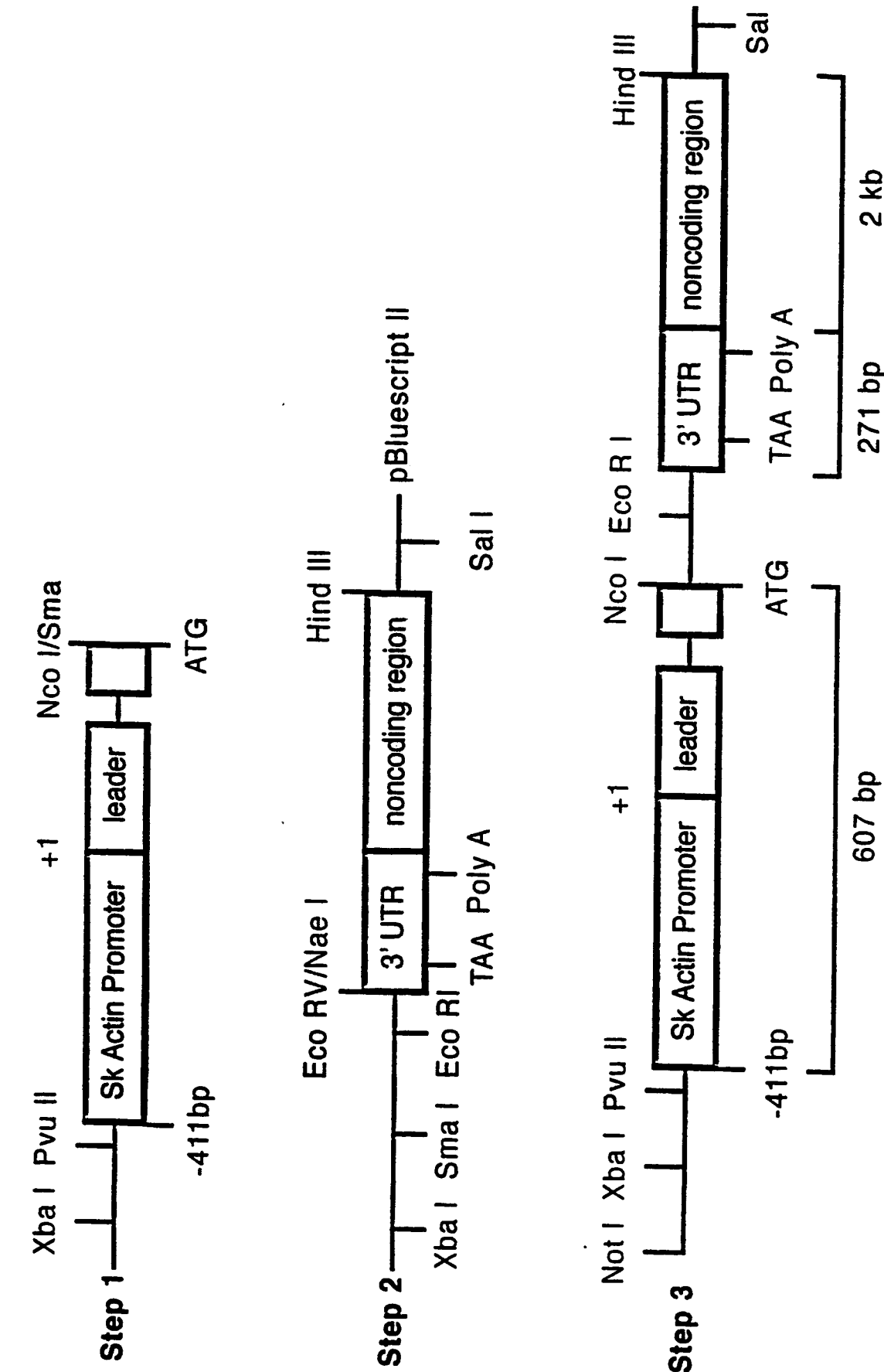
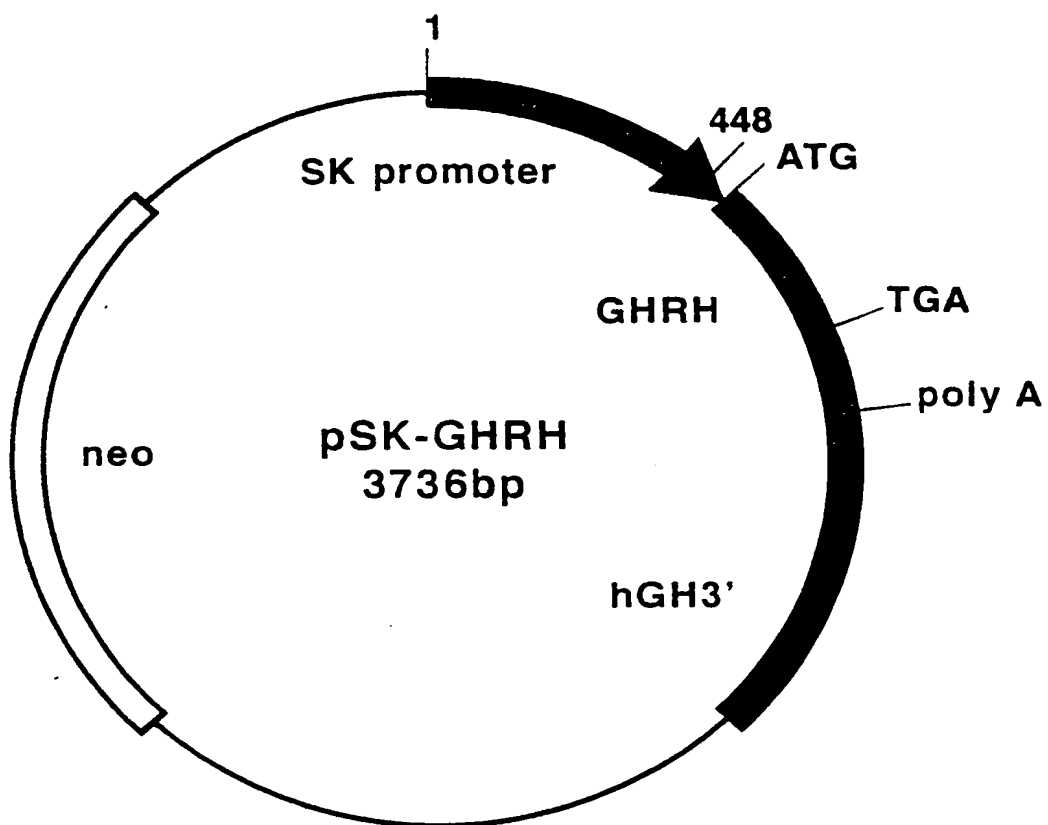


Fig.1

# MYOGENIC VECTOR SYSTEM



**Fig. 2**

**Fig. 3**

## Codon Frequency

human\_high.doc

**Codon usage for human (highly expressed) genes 1/24/91**

AmAcid	Codon	Number	/1000	Fraction	AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	905.00	18.76	0.24	Trp	TGG	652.00	13.51	1.00
Gly	GGA	525.00	10.88	0.14	End	TGA	109.00	2.26	0.55
Gly	GGT	441.00	9.14	0.12	Cys	TGT	325.00	6.74	0.32
Gly	GGC	1867.00	38.70	0.50	Cys	TGC	706.00	14.63	0.68
Glu	GAG	2420.00	50.16	0.75	End	TAG	42.00	0.87	0.21
Glu	GAA	792.00	16.42	0.25	End	TAA	46.00	0.95	0.23
Asp	GAT	592.00	12.27	0.25	Tyr	TAT	360.00	7.46	0.26
Asp	GAC	1821.00	37.75	0.75	Tyr	TAC	1042.00	21.60	0.74
Val	GTG	1866.00	38.68	0.64	Leu	TTG	313.00	6.49	0.06
Val	GTA	134.00	2.78	0.05	Leu	TTA	76.00	1.58	0.02
Val	GTT	198.00	4.10	0.01	Phe	TTT	336.00	6.96	0.20
Val	GTC	728.00	15.09	0.25	Phe	TTC	1377.00	28.54	0.80
Ala	GCG	652.00	13.51	0.17	Ser	TCG	325.00	6.74	0.09
Ala	GCA	488.00	10.12	0.13	Ser	TCA	165.00	3.42	0.05
Ala	GCT	654.00	13.56	0.17	Ser	TCT	450.00	9.33	0.13
Ala	GCC	2057.00	42.64	0.53	Ser	TCC	958.00	19.86	0.28
Arg	AGG	512.00	10.61	0.18	Arg	CGG	611.00	12.67	0.21
Arg	AGA	298.00	6.18	0.10	Arg	CGA	183.00	3.79	0.06
Ser	AGT	354.00	7.34	0.10	Arg	CGT	210.00	4.35	0.07
Ser	AGC	1171.00	24.27	0.34	Arg	CGC	1086.00	22.51	0.37
Lys	AAG	2117.00	43.88	0.82	Gln	CAG	2020.00	41.87	0.88
Lys	AAA	471.00	9.76	0.18	Gln	CAA	283.00	5.87	0.12
Asn	AAT	314.00	6.51	0.22	His	CAT	234.00	4.85	0.21
Asn	AAC	1120.00	23.22	0.78	His	CAC	870.00	18.03	0.79
Met	ATG	1077.00	22.32	1.00	Leu	CTG	2864.00	59.78	0.58
Ile	ATA	88.00	1.82	0.05	Leu	CTA	166.00	3.44	0.03
Ile	ATT	315.00	6.53	0.18	Leu	CTT	238.00	4.93	0.05
Ile	ATC	1369.00	28.38	0.17	Leu	CTC	1276.00	26.45	0.26
Thr	ACG	405.00	8.40	0.15	Pro	CCG	482.00	9.99	0.17
Thr	ACA	373.00	7.73	0.14	Pro	CCA	456.00	9.45	0.16
Thr	ACT	358.00	7.42	0.14	Pro	CCT	568.00	11.77	0.19
Thr	ACC	1502.00	31.13	0.57	Pro	CCC	1410.00	29.23	0.48

**Fig. 4**

pRH0964 (from 539 to 766) is the wild type human GHRH sequence  
translate \_7\_9 is the GHRH sequence with optimal human codons

LOCUS pRH0964 3697 bp dsDNA Circular  
DEFINITION pVC0289 with SK-GHRH (NotI/salI) fragment from pMVG3X

Quality: 1577 Length: 228 Match display thresholds for the alignment(s):  
Ratio: 6.917 Gaps: 0 | = IDENTITY  
Percent Similarity: 83.772 Percent Identity: 83.772 : = 5  
TOP ROW (BASES 539 TO 766) = SEQ ID NO. 1 . = 1  
BOTTOM ROW (BASES 1 TO 228) = SEQ ID NO.2

539	ATGGTGCTCTGGGTGTTCTTCTTTGTGATCCTCACCCCTCAGCAACAGCTC	588
1		
	ATGGTGCTGTTGTTCTTCTTCTGATCCTGACCCTGAGCAACAGCAG	50
589	CCACTGCTCCCCACCTCCCTTTGACCCCTCAGGATGCGGCGGTATGCAG	638
51	CCACTGCAGCCCCCCCCCTGACCCCTGCGCATGCGCCGCTACGCCG	100
639	ATGCCATCTTCACCAACAGCTACCGGAAGGTGCTGGGCCAGCTGTCCGCC	688
101	ACGCCATCTTCACCAACAGCTACCGCAAGGTGCTGGGCCAGCTGAGCGCC	150
689	CGCAAGCTGCTCCAGGACATCATGAGCAGGCAGCAGGAGAGCAACCA	738
151	CGCAAGCTGCTGCAGGACATCATGAGCCGCCAGCAGGCGGAGAGCAACCA	200
739	AGAGCGAGGAGCAAGGGCACGGCTTTAA	766
201	GGAGCGCGGCGCCGCGCCGCGCTGTGA	228

**Fig. 5**

pSK-GHRH NotI/SalI fragment (SK promoter, GHRHcDNA, hGH3')  
Coding region for GHRH in bold.

SEQ ID NO. 4

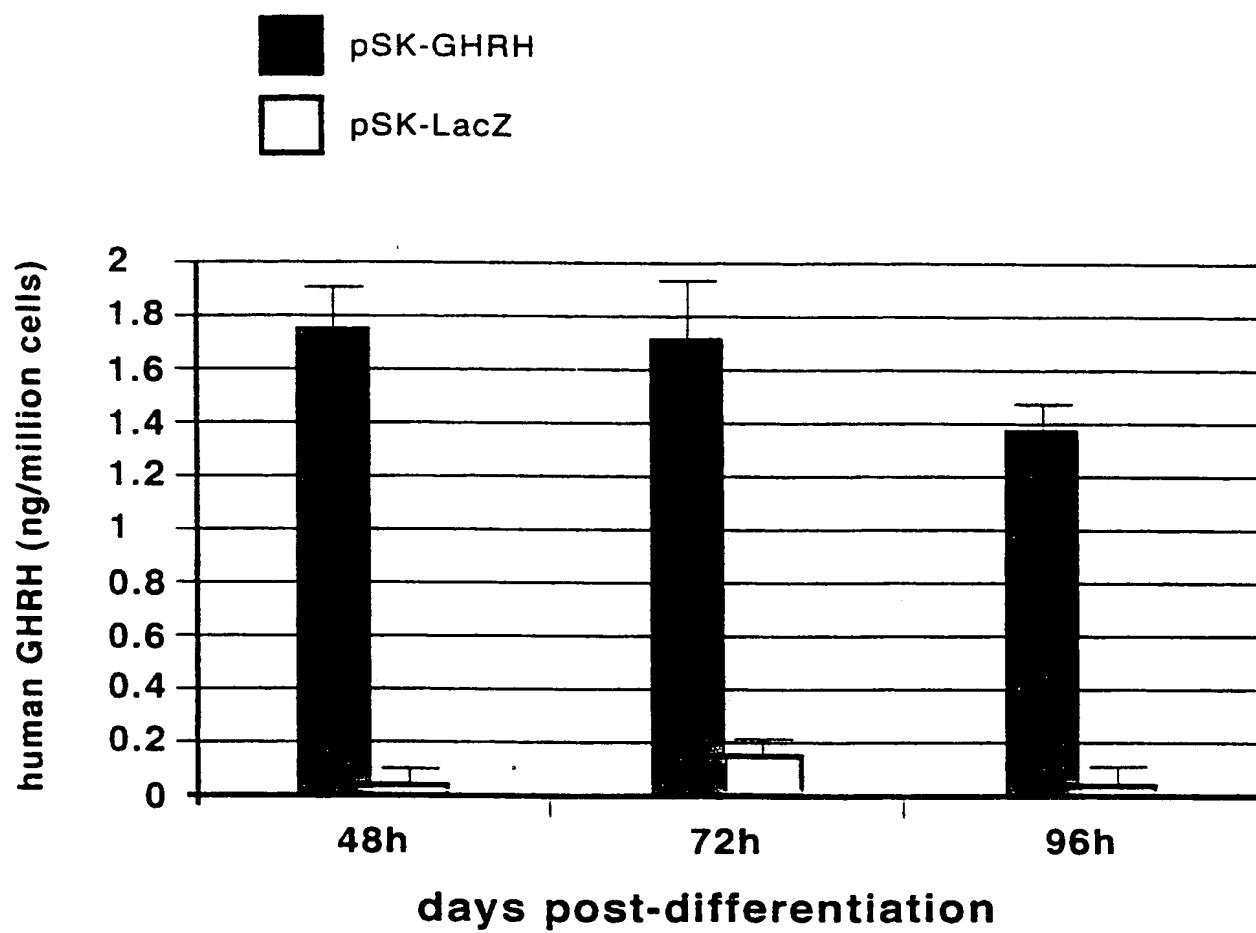
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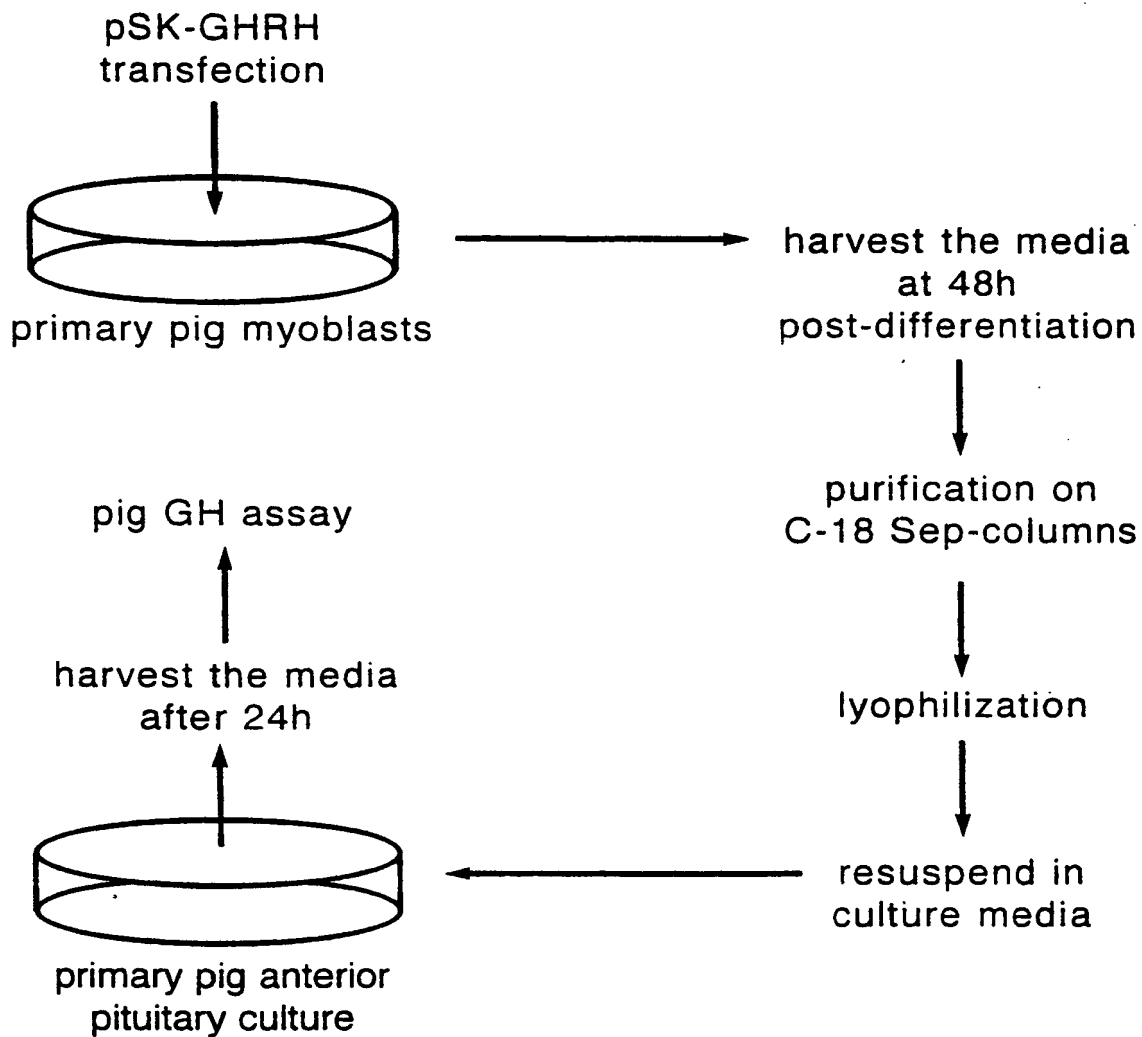
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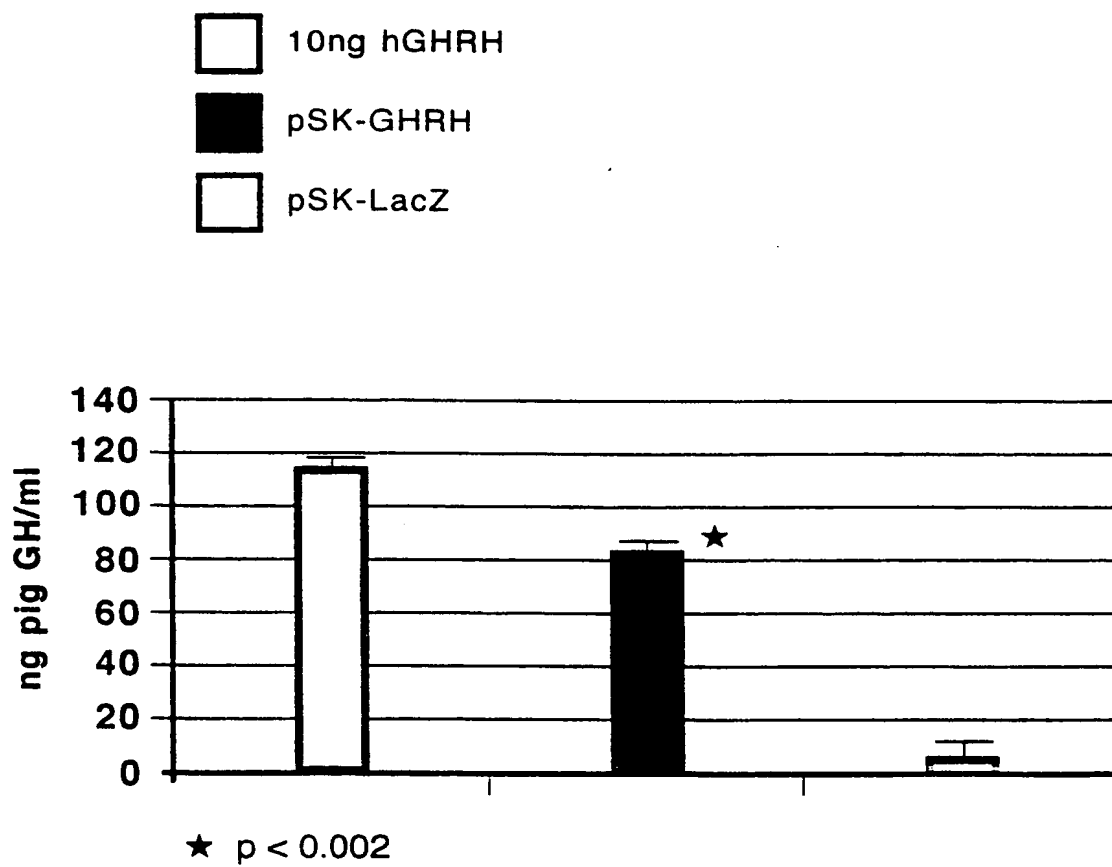
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Fig. 6



**Fig. 7**

**Fig. 8**

**Fig. 9**

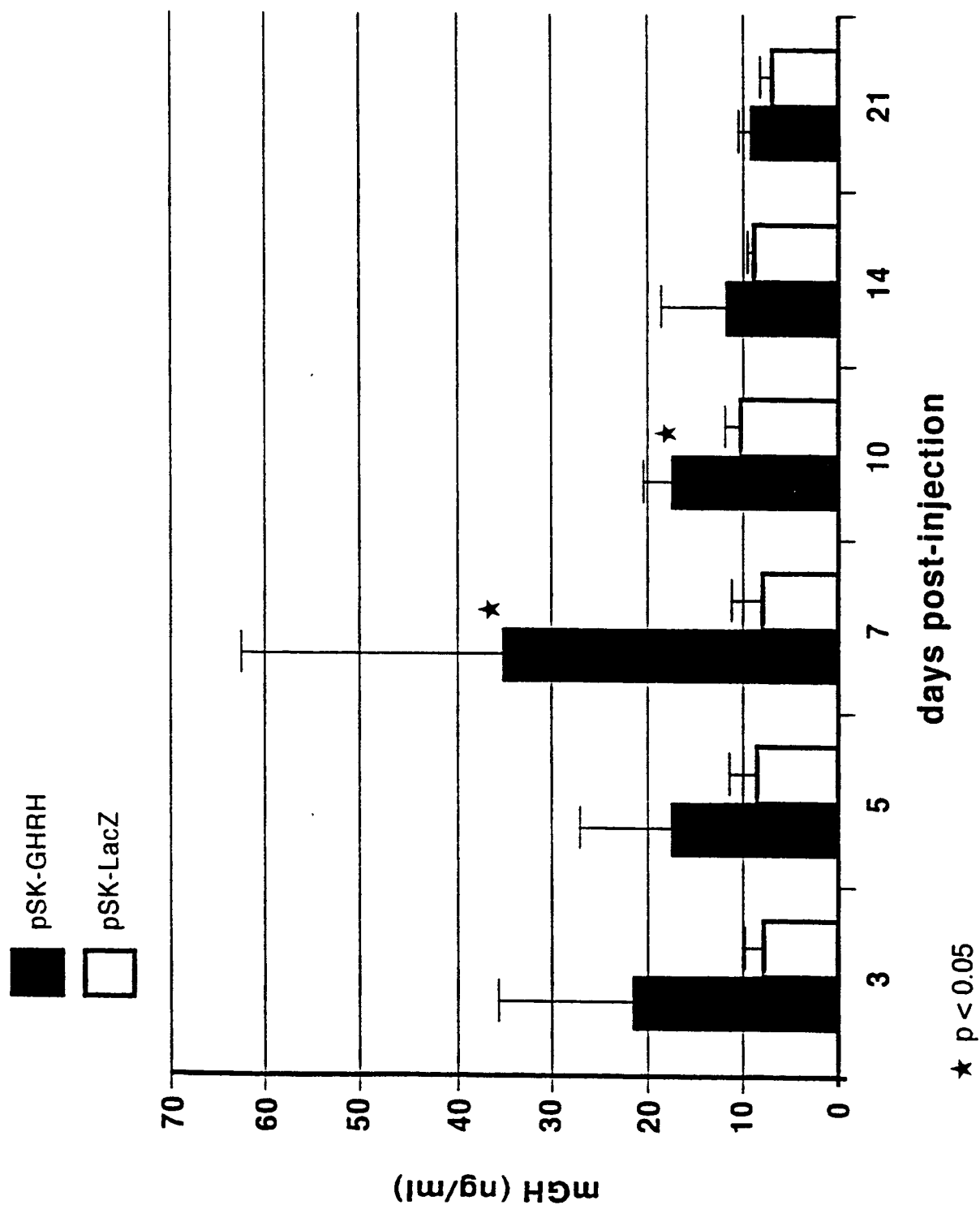
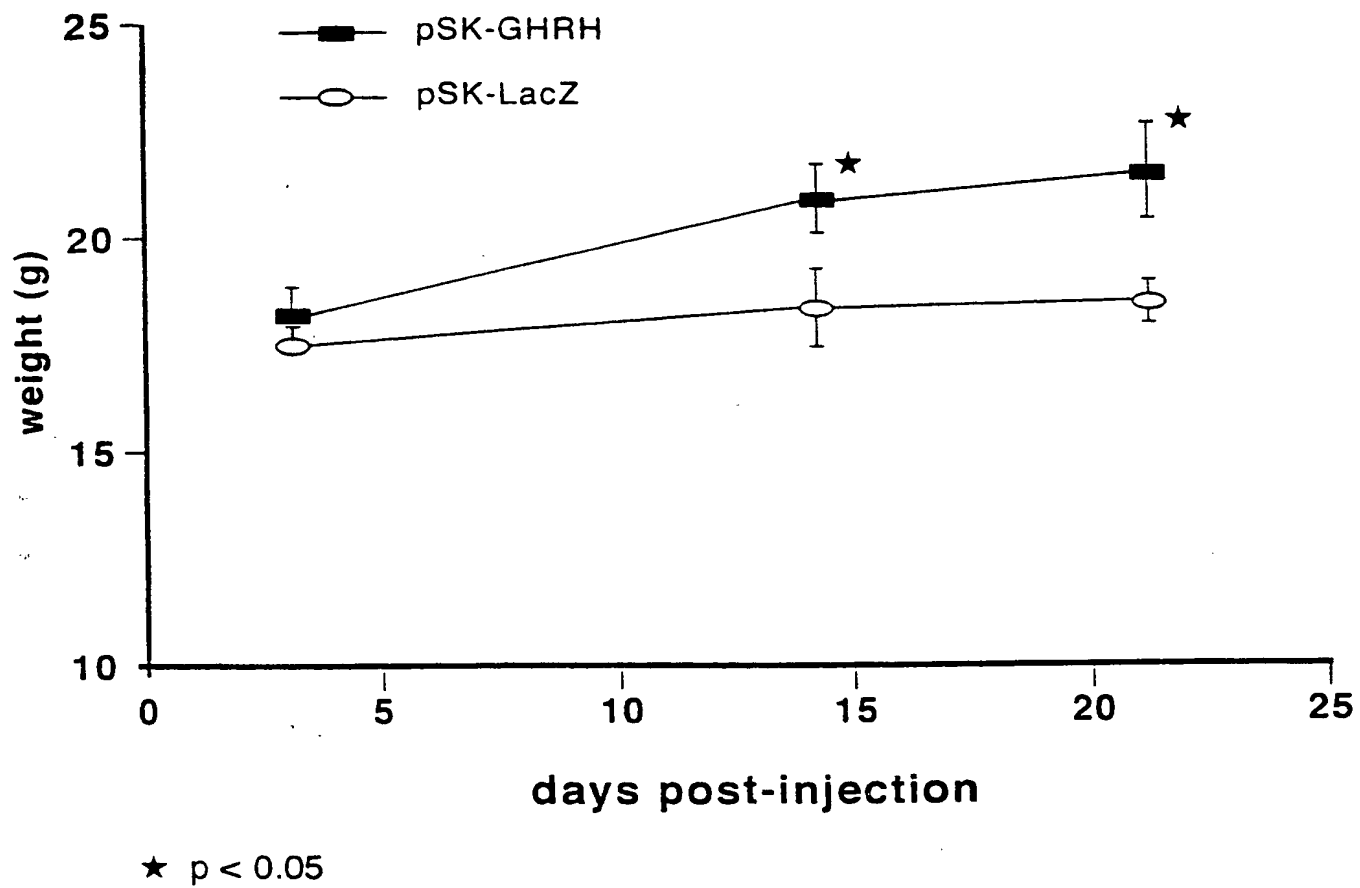
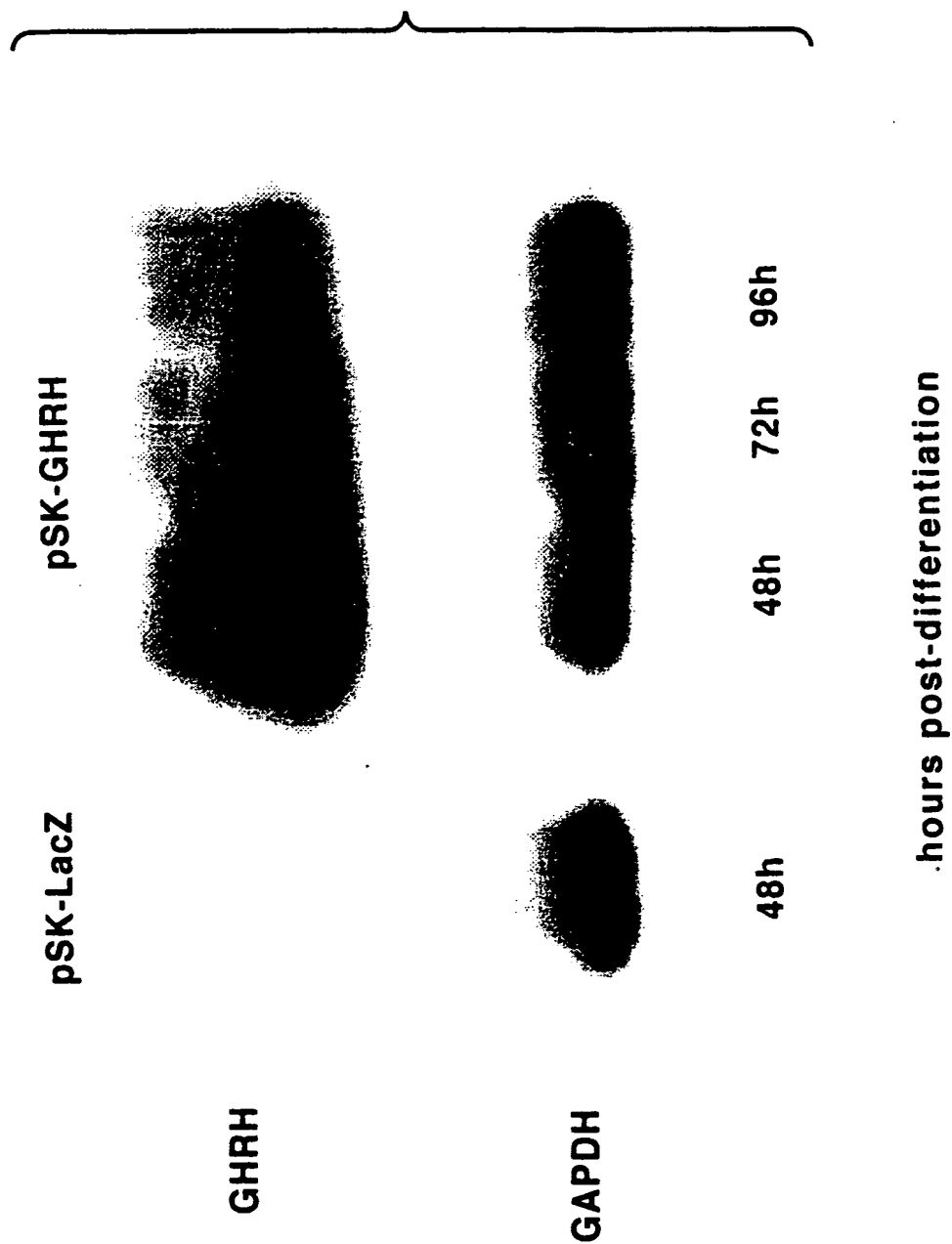
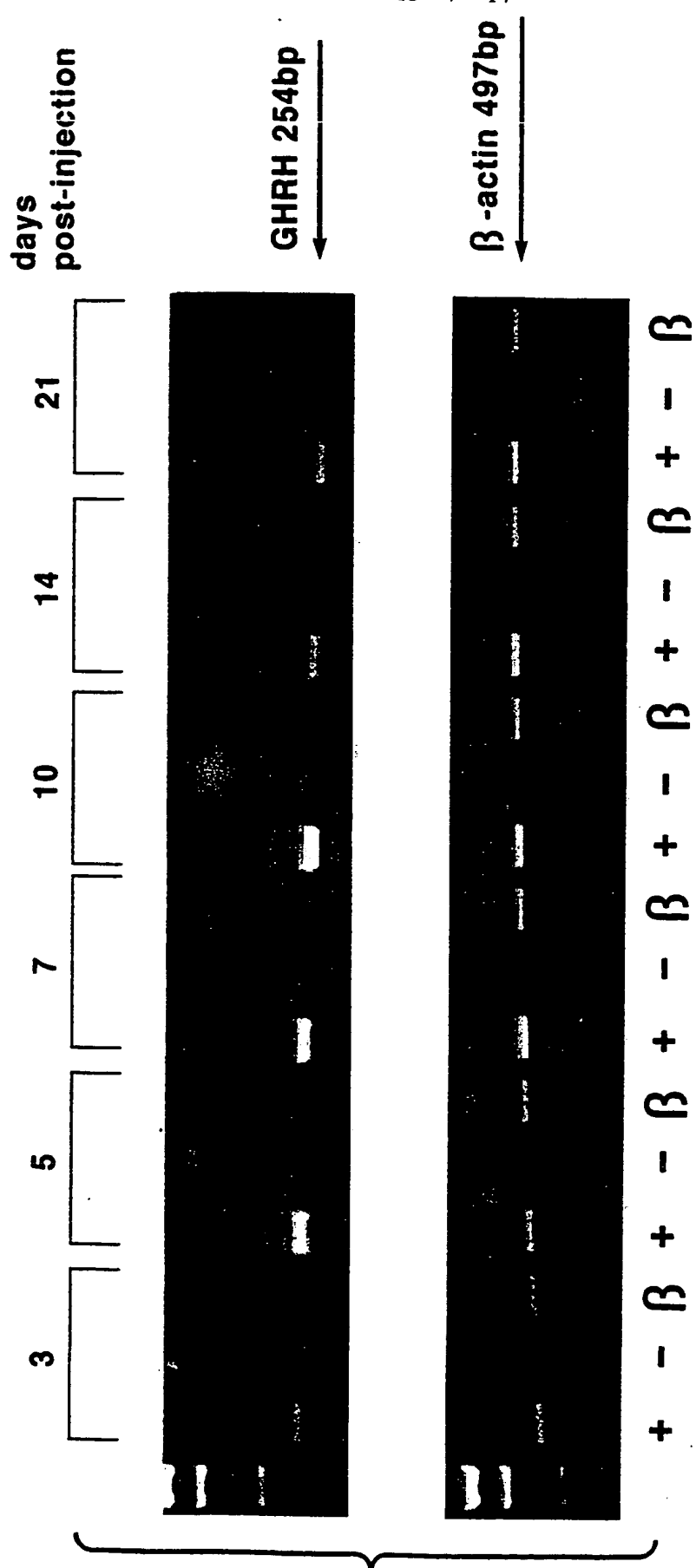


Fig. 10

**Fig. 11**



**Fig. 12**



+ = pSK-GHRH + RT  
 - = pSK-GHRH - RT  
 $\beta$  = pSK-LacZ + RT

Fig. 13

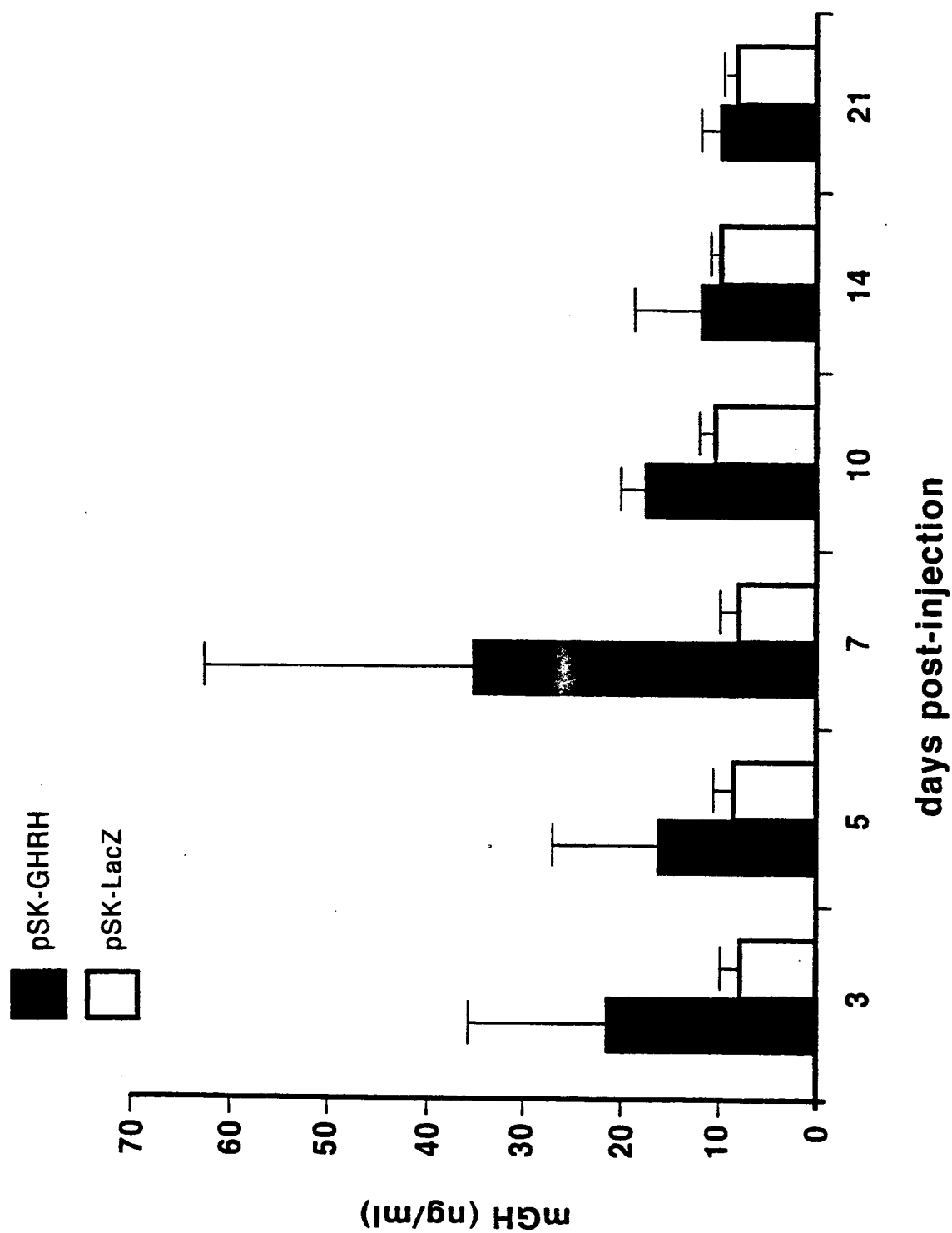


Fig. 14



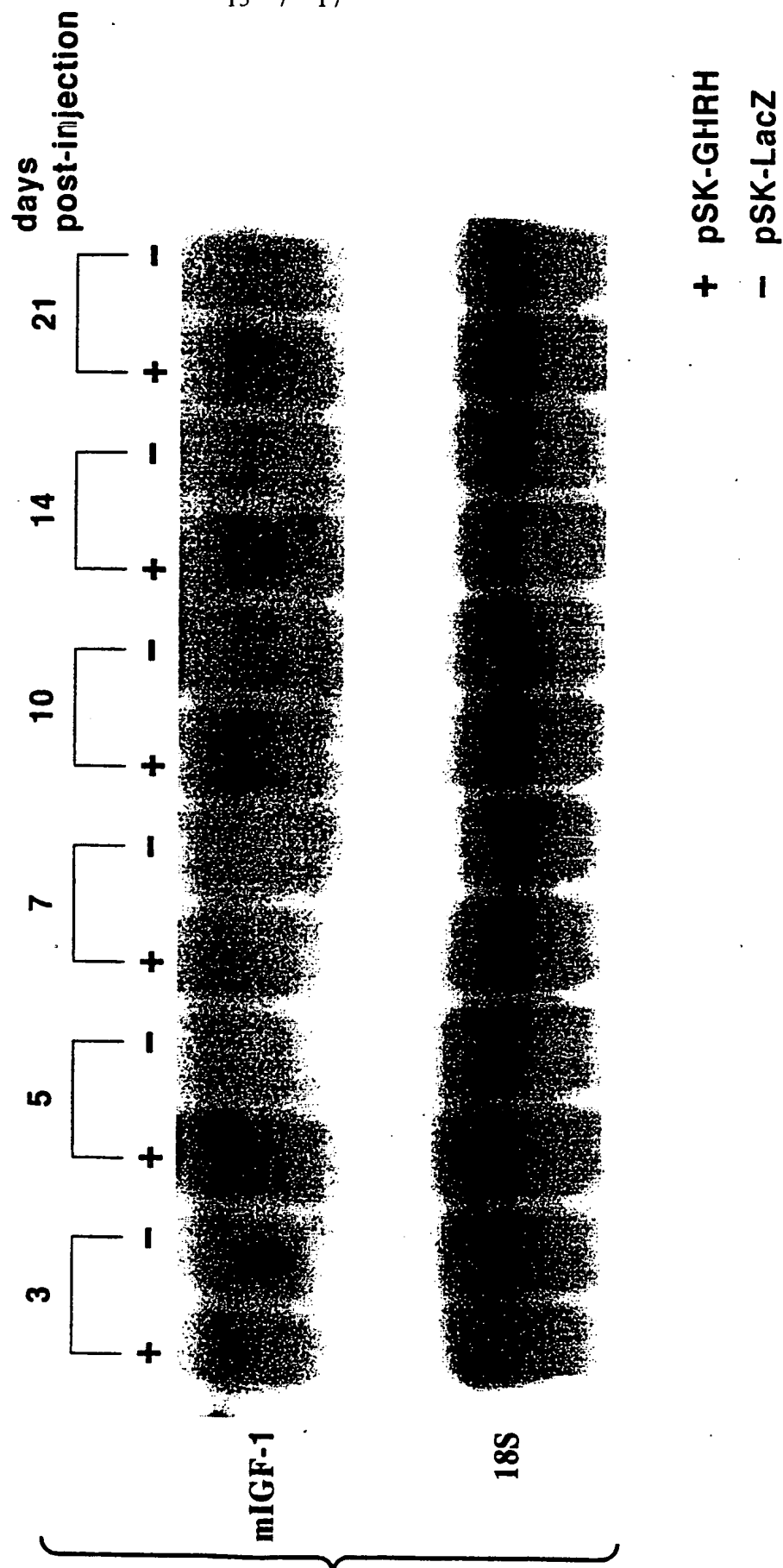
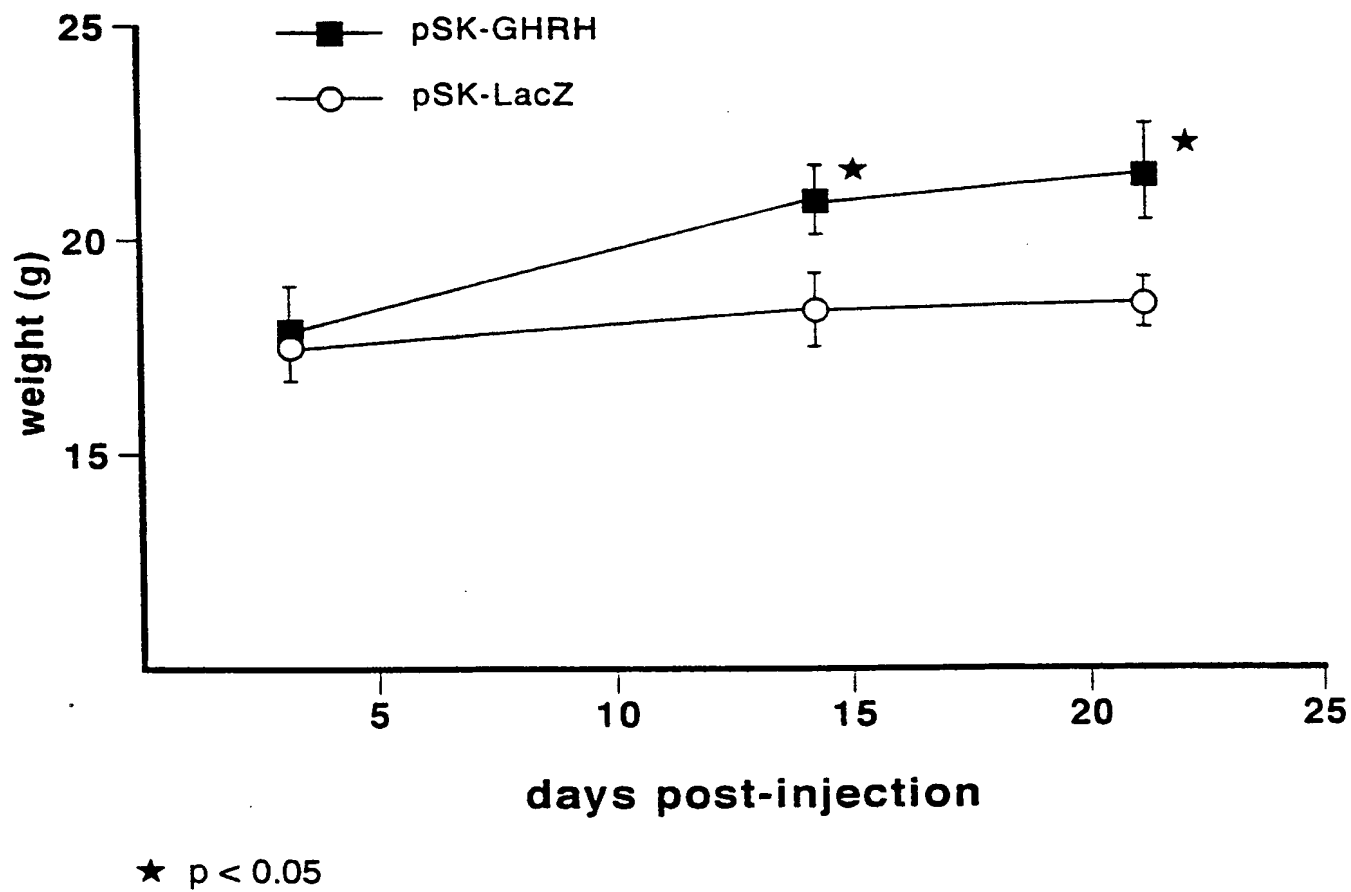


Fig. 15

**Fig. 16**

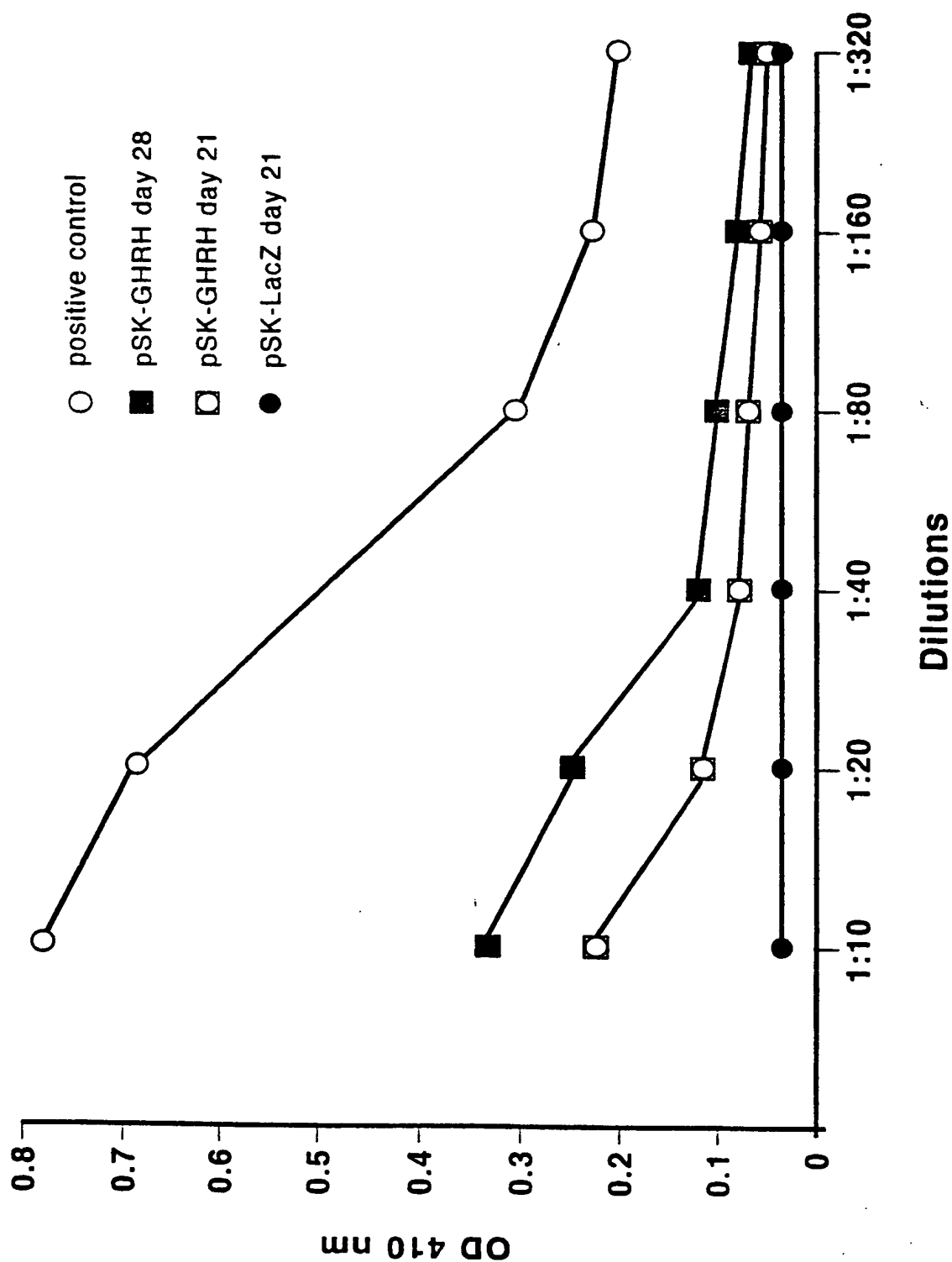


Fig. 17

## SEQUENCE LISTING

<110> Robert J. Schwartz  
 Ruxandra Draghia-Akli  
 Xuyang Li  
 Eric Eastman

<120> GHRH EXPRESSION SYSTEM AND METHODS OF  
 USE

<130> 225/252-PCT

<150> 60/053,609

<151> 1997-07-24

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<213> pSK-GHRH

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taccggaagg	tgctgggcca	gctgtccgcc	cgcaagctgc	tccaggacat	catgagcagg	180
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taccgcaagg	tgctgggcca	gctgagcgcc	cgcaagctgc	tgaggacat	catgagccgc	180
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&lt;211&gt; 10

&lt;212&gt; DNA

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&lt;220&gt;

&lt;223&gt; The letter "w" stands for a or t.

&lt;400&gt; 3

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10

&lt;210&gt; 4

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&lt;212&gt; DNA

&lt;213&gt; pSK-GHRH

&lt;400&gt; 4

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&lt;211&gt; 125

&lt;212&gt; PRT

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&lt;400&gt; 5

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 Gly Leu Tyr Ser Val Ala Leu Leu Glu Gly Leu Tyr Gly Leu Asn Leu  
 35 40 45  
 Glu Ser Glu Arg Ala Leu Ala Ala Arg Gly Leu Tyr Ser Leu Glu Leu  
 50 55 60  
 Glu Gly Leu Asn Ala Ser Pro Ile Leu Glu Met Glu Thr Ala Ser Asn  
 65 70 75 80  
 Ala Arg Gly Gly Leu Asn Gly Leu Asn Gly Leu Tyr Gly Leu Ala Arg  
 85 90 95  
 Gly Ala Ser Asn Gly Leu Asn Gly Leu Gly Leu Asn Gly Leu Tyr Ala  
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&lt;211&gt; 125

&lt;212&gt; PRT

&lt;213&gt; pSK-GHRH

&lt;400&gt; 6

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 Gly Leu Tyr Ser Val Ala Leu Leu Glu Gly Leu Tyr Gly Leu Asn Leu  
 35 40 45  
 Glu Ser Glu Arg Ala Leu Ala Ala Arg Gly Leu Tyr Ser Leu Glu Leu  
 50 55 60

Glu Gly Leu Asn Ala Ser Pro Ile Leu Glu Met Glu Thr Ser Glu Arg  
 65 70 75 80  
 Ala Arg Gly Gly Leu Asn Gly Leu Asn Gly Leu Tyr Gly Leu Ala Arg  
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 Gly Ala Ser Asn Gly Leu Asn Gly Leu Gly Leu Asn Gly Leu Tyr Ala  
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 Leu Ala Ala Arg Gly Val Ala Leu Ala Arg Gly Leu Glu  
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&lt;211&gt; 125

&lt;212&gt; PRT

&lt;213&gt; pSK-GHRH

&lt;400&gt; 7

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 20 25 30  
 Gly Leu Tyr Ser Ile Leu Glu Leu Glu Gly Leu Tyr Gly Leu Asn Leu  
 35 40 45  
 Glu Ser Glu Arg Ala Leu Ala Ala Arg Gly Leu Tyr Ser Leu Glu Leu  
 50 55 60  
 Glu Gly Leu Asn Ala Ser Pro Ile Leu Glu Met Glu Thr Ala Ser Asn  
 65 70 75 80  
 Ala Arg Gly Gly Leu Asn Gly Leu Asn Gly Leu Tyr Gly Leu Ala Arg  
 85 90 95  
 Gly Ala Ser Asn Gly Leu Asn Gly Leu Gly Leu Asn Gly Leu Tyr Ala  
 100 105 110  
 Leu Ala Leu Tyr Ser Val Ala Leu Ala Arg Gly Leu Glu  
 115 120 125

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&lt;211&gt; 120

&lt;212&gt; PRT

&lt;213&gt; pSK-GHRH

SUBSTITUTE SHEET (RULE 26)

&lt;400&gt; 8

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His Glu Thr His Arg Thr His Arg Ala Ser Asn Thr Tyr Arg Ala Arg
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Gly Leu Tyr Ser Leu Glu Leu Glu Ser Glu Arg Gly Leu Asn Leu Glu
      35              40              45

Thr Tyr Arg Ala Leu Ala Ala Arg Gly Leu Tyr Ser Val Ala Leu Ile
      50              55              60

Leu Glu Gly Leu Asn Ala Ser Pro Ile Leu Glu Met Glu Thr Ala Ser
      65              70              75              80

Asn Leu Tyr Ser Gly Leu Asn Gly Leu Tyr Gly Leu Ala Arg Gly Ile
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Leu Glu Gly Leu Asn Gly Leu Gly Leu Asn Ala Arg Gly Ala Leu Ala
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Ala Arg Gly Leu Glu Ser Glu Arg
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&lt;211&gt; 125

&lt;212&gt; PRT

&lt;213&gt; pSK-GHRH

&lt;400&gt; 9

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Gly Leu Tyr Ser Val Ala Leu Leu Glu Gly Leu Tyr Gly Leu Asn Leu
      35              40              45

Glu Ser Glu Arg Ala Leu Ala Ala Arg Gly Leu Tyr Ser Leu Glu Leu
      50              55              60

Glu Gly Leu Asn Ala Ser Pro Ile Leu Glu Met Glu Thr Ala Ser Asn
      65              70              75              80

Ala Arg Gly Gly Leu Asn Gly Leu Asn Gly Leu Tyr Gly Leu Ala Arg
      85              90              95

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Glu Ser Glu Arg Ala Leu Ala Ala Arg Gly Leu Tyr Ser Leu Glu Leu  
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Glu Gly Leu Asn Ala Ser Pro Ile Leu Glu Met Glu Thr Ser Glu Arg  
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Ala Arg Gly Gly Leu Asn Gly Leu Asn Gly Leu Tyr Gly Leu Ser Glu  
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Leu Ala

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&lt;213&gt; pSK-GHRH

&lt;400&gt; 12

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&lt;212&gt; DNA

&lt;213&gt; pSK-GHRH

&lt;400&gt; 13

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20



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/85, 15/86, 15/62, 15/12, 5/10,</b> <b>C07K 14/60, A01K 67/027, A61K 47/32,</b> <b>48/00</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 99/05300</b>  <b>(43) International Publication Date:</b> 4 February 1999 (04.02.99)
<b>(21) International Application Number:</b> PCT/US98/15434  <b>(22) International Filing Date:</b> 24 July 1998 (24.07.98)  <b>(30) Priority Data:</b> 60/053,609                      24 July 1997 (24.07.97)                      US 60/062,608                      20 October 1997 (20.10.97)                      US  <b>(71) Applicant (for all designated States except US):</b> GEN- EMEDICINE, INC. [US/US]; 8301 New Trails Drive, The Woodlands, TX 77381-4248 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SCHWARTZ, Robert, J. [US/US]; 4019 Marlowe, Houston, TX 77005 (US). DRAGHIA-AKLI, Ruxandra [RO/US]; 2255 Braeswood Park Drive #159, Houston, TX 77030 (US). LI, Xuyang [CN/US]; 2111 Holly Hail #310, Houston, TX 77054 (US). EASTMAN, Eric, M. [US/US]; 7465 Mink Hollow Road, Highland, MD 20777 (US).  <b>(74) Agents:</b> WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>  <b>(88) Date of publication of the international search report:</b> 15 April 1999 (15.04.99)
<b>(54) Title:</b> GHRH EXPRESSION SYSTEM AND METHODS OF USE  <b>(57) Abstract</b>  This invention relates to gene delivery and expression, including gene therapy, by using vectors which establish controlled expression of recombinant GHRH genes within tissues at certain levels. The expression vectors of the present invention can also be regulated by a regulatory system and/or constructed with a coating. The invention concerns the use of plasmid cDNA containing nucleotide sequences encoding species-specific GHRH polypeptides and teaches expression of these polypeptides in a variety of animal species and animal tissues to provide stimulation of GH at physiological levels, sufficient to enhance growth and improve the immune systems of agricultural and other domesticated species, including household pets. The invention also concerns a novel method for reducing the rate of proteolytic degradation of secreted GHRH.		

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EE	Estonia						

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/15434

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 C12N15/86 C12N15/62 C12N15/12 C12N5/10  
C07K14/60 A01K67/027 A61K47/32 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 09236 A (BAYLOR COLLEGE MEDICINE) 13 May 1993  see abstract see page 1, line 18 - line 23 see page 4, line 15 - line 20 see page 5, line 20 - line 28 see page 8, line 29 - line 31 see page 10, line 27 - page 11, line 26 see page 12, line 25 - line 27 see example 8 see claims 1,8,17,20,27,46	1,2,5-7, 12-18, 21-26, 28-36, 38-41, 45,46
Y	---	3,8-11, 19,20, 27,31
	- / - -	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

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 "&" document member of the same patent family

Date of the actual completion of the international search

9 November 1998

Date of mailing of the international search report

25.02.99

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Authorized officer

Galli, I

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/15434

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	HAMMER R.E. ET AL.: "Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth." NATURE, vol. 312, 30 May 1985, pages 413-416, XP002083746 see the whole document ---	1,2, 21-24
X	EP 0 134 085 A (SALK INST FOR BIOLOGICAL STUDI) 13 March 1985 see abstract see page 12, line 18 - page 13, line 15 see table 1 ---	1,2
A	---	3-36, 38-41, 45,46
X	US 4 857 470 A (MORAIS CRAVADOR ALFREDO J ET AL) 15 August 1989 see abstract see page 2, column 1, line 64 - column 2, line 3 ---	1
Y	---	3
X	EP 0 509 831 A (LILLY CO ELI) 21 October 1992 see abstract ---	1
X	EP 0 459 747 A (LILLY CO ELI) 4 December 1991 see page 1-8 ---	1
A	WO 97 22625 A (INDIANA UNIVERSITY FOUNDATION ;PESCOVITZ ORA H (US); WILLIAMS DAVI) 26 June 1997 see abstract see figure 15 see page 14 see figure 7 ---	1-36, 38-41, 45,46
Y	WO 96 21470 A (ROLLAND ALAIN ;GENEMEDICINE INC (US); MUMPER RUSSELL J (US)) 18 July 1996 cited in the application see abstract ---	19,20, 27,31
P,X	DRAGHIA-AKLI R ET AL: "ENHANCED GROWTH BY ECTOPIC EXPRESSION OF GROWTH HORMONE RELEASING HORMONE USING AN INJECTABLE MYOGENIC VECTOR" NATURE BIOTECHNOLOGY, vol. 15, no. 12, November 1997, pages 1285-1289, XP002060954 see the whole document ---	1-6, 8-18,25, 29,36, 38-41,45
	---	-/--

# INTERNATIONAL SEARCH REPORT

Inter national Application No  
PCT/US 98/15434

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	<p>WO 98 24922 A (BAYLOR COLLEGE MEDICINE ;GENEMEDICINE INC (US)) 11 June 1998 see claims 1,7</p> <p>-----</p>	8-11

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 15434

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 25-28,32-36,38-41,45,46 and (as far as methods in vivo are envisaged claims 29-31 are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

(1-35)- complete; (36,38-41,45,46)- partial

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 1. Claims: (1-35) - complete; (36,38-41,45,46) - partial

A vector comprising a 5' regulatory region, a nucleic acid encoding GHRH inserted in a linker sequence, and a 3' flanking region.

In particular, a vector for expressing human GHRH (as defined in Seq. ID 2) in a cell, comprising a skeletal alpha-actin gene promoter and a 3'-UTR from the human Growth Hormone gene.

A formulation for delivery of said vector to a cell comprising 5% polyvinylpyrrolidone. A method for the delivery and expression of said vector in vertebrate cells. Transgenic animals carrying this vector.

## 2. Claims: (42-44) - complete; (36) - partial

A method for the delivery and expression of GHRH in a non-human vertebrate animal using an adenovirus or adeno-associated virus carrying a GHRH cDNA linked to a promoter and a 3'-flanking region.

## 3. Claims: (36-41,45,46) - partial

A method for the delivery and expression of vectors carrying a GHRH gene of vertebrate, wherein the gene is encoded by Seq. ID 5.

## 4. Claims: (36-41,45,46) - partial

Idem as subject matter 4, but limited to seq. ID 6.

## 5. Claims: (36-41,45,46) - partial

Idem as subject matter 4, but limited to seq. ID 7.

## 6. Claims: (36-41,45,46) - partial

Idem as subject matter 4, but limited to seq. ID 8.

## 7. Claims: (36-41,45,46) - partial

Idem as subject matter 4, but limited to seq. ID 9.

## 8. Claims: (47-49) - complete; (36-41,45,46) - partial

A method for the delivery and expression of vectors carrying

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

a chimeric synthetic cDNA encoding amino acids 1-15 of mouse-specific GHRH and amino acids 16-44 of another vertebrate's GHRH.

Said method, wherein the other vertebrate's GHRH is defined by Seq. IDs 5-9 and a chiumera is defined by seq. ID. 11.

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